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Choline metabolism and phosphatidylcholine biosynthesis in cultured rat hepatocytes

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1. Adult rat hepatocytes were isolated by collagenase perfusion and were maintained in monolayer culture for 24h. 2. Choline metabolism and phosphatidylcholine biosynthesis were studied in these cells by performing pulse–chase studies at physiological concentrations (1–40μM) of (*Me*-^{3}H)-labelled or unlabelled choline in the culture medium. 3. During the 15min pulse incubation, choline entering the cells was rapidly phosphorylated to phosphocholine or oxidized to betaine. Low concentrations of choline in the medium decreased the relative amount of choline oxidized. 4. During the 3h chase period, the radioactivity in the phosphocholine pool was transferred to phosphatidylcholine. Very little radioactivity was associated with CDP-choline. These results provide good evidence that the rate-limiting step for phosphatidylcholine biosynthesis in these cultured hepatocytes is the conversion of phosphocholine into CDP-choline. Similar results were obtained for all concentrations of choline in the culture medium. 5. Cellular concentrations of phosphocholine were unaffected by the concentration of choline (1–40μM) in the medium. 6. The majority of the label associated with betaine was secreted into the culture medium during the chase incubation. 7. From the pulse–chase studies, and the cellular phosphocholine concentrations, it was possible to estimate the rate of phosphatidylcholine biosynthesis (2.2, 2.8, 3.1 and 3.7nmol/min per g wet weight of cells cultured in 1, 5, 10 and 40μM-choline respectively for up to 4.25h).

The major pathway for the biosynthesis of phosphatidylcholine via CDP-choline was elucidated in the 1950s (Kennedy, 1962). Subsequently, the methylation of phosphatidylethanolamine to yield phosphatidylcholine was demonstrated in liver (Bremer & Greenberg, 1961). Two decades have now elapsed, but our understanding of the regulation of phosphatidylcholine biosynthesis is still at an elementary level (Vance & Choy, 1979).

The liver is a unique tissue for the study of control of choline metabolism and phosphatidylcholine biosynthesis. This is one of the few tissues in which choline can be either phosphorylated or oxidized to betaine. Secondly, phosphatidylcholine synthesis is required for the cellular membranes as appears to be true in all tissues. In addition, the liver is a major source for the phosphatidylcholine of plasma lipoproteins and bile. What mechanisms are used to direct choline either toward oxidation to betaine or phosphatidylcholine biosynthesis? What regulates the rate of phosphatidylcholine synthesis and decides whether the lipid should be directed toward membranes, lipoproteins or bile? Are there control mechanisms that integrate the rate of phosphatidylcholine biosynthesis via the CDP-choline and methylation pathway? The answers to these questions are not available.

We have initiated studies on the metabolism of choline and the biosynthesis of phosphatidylcholine in isolated rat hepatocytes maintained in a monolayer culture. This system provides all of the advantages of tissue culture, yet reflects to a large extent the function of liver cells *in vivo*. In addition, the liver is a major source for the isolation of the enzymes of choline metabolism and phosphatidylcholine synthesis. Thus, the characteristics *in vitro* of the enzymes can be readily correlated with their function in the cultured cells.

The metabolism of choline has been studied to a limited extent in isolated hepatocytes (Sundler & Åkesson, 1975). Although these studies were performed at high concentrations of choline in the medium, the results provided some of the first evidence that the rate-limiting step for phosphatidylcholine biosynthesis is catalysed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15).

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We now report on choline metabolism in cultured rat hepatocytes at concentrations in the medium that are similar to rat serum (Ziesel *et al.*, 1980). The results suggest that the concentration of choline is an important factor in the control of choline oxidation or phosphorylation. Secondly, the results strongly support the proposal that CTP:phosphocholine cytidylyltransferase catalyses the rate-limiting step for phosphatidylcholine biosynthesis in rat liver.

Materials and methods

Materials

Arginine- and choline-free Dulbecco's modified Eagle's medium was custom made by Grand Island Biological Co., Calgary, Alberta, Canada. Foetal calf serum was purchased from Wildlife Serums, Calgary, Alberta, Canada. Insulin and collagenase type IV were purchased from Sigma, St. Louis, MO, U.S.A., and [*Me*-^{3}H]choline chloride was obtained from Amersham, Oakville, Ontario, Canada. Contur tissue culture dishes were from Lux Scientific Corp., Newbury Parks, CA, U.S.A.

Isolation and culture of hepatocytes

Female Wistar rats (approx. 160g) were purchased from the University of British Columbia animal unit and were fed a purina rat chow and water diet *ad libitum* before all experiments. Hepatocytes were routinely isolated between 09:00 and 11:00h by a modification of the collagenase perfusion method described by Davis *et al.* (1979). The final preparation of dispersed hepatocytes was resuspended in arginine-free Dulbeco's modified Eagle's medium with 20% foetal calf serum, insulin (10μg/ml), 0.4mM-ornithine, streptomycin sulphate (100μg/ml), penicillin G (100units/ml) and 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4.

Cells were dispersed into 60mm plastic culture dishes at 1×10^6cells/ml (3ml/dish) and incubated at 37°C in air/CO_2 (19:1).

Pulse–chase studies on the metabolism of choline

After 24h incubation, 40 dishes of cells were washed twice with 4ml of choline-free medium that contained 5% dialysed foetal calf serum. The cells were pre-incubated for 1h in 2ml of medium that contained 1, 5, 10 or 40μM-choline (10 dishes per concentration). After the pre-incubation, the medium in each dish was replaced with the same medium that now also contained 10μCi of [*Me*-^{3}H]choline/ml. The cells were incubated for 15min. At the end of the pulse period, the cells were washed three times with 4ml of choline-free medium to remove any unincorporated [*Me*-^{3}H]choline. Chase medium with the appropriate concentration of choline was added to each dish of cells and the incubation was continued. Incubations were stopped at the start and after 0.5, 1, 2 and 3h of the chase period (two dishes at each concentration per time point). This was done by removal of the chase medium, washing the cells twice with ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954) and scraping the cells into ice-cold methanol/water (5:4, v/v). Harvesting the cells by this method took approx. 1min. Lipids were extracted by the method of Folch *et al.* (1957). After separation of the phases, the top phase was removed and the bottom phase was washed three times with more top phase. Choline metabolites secreted into the medium were also separated into water-soluble and lipid-soluble fractions by extraction of the chase medium by the method of Folch *et al.* (1957).

Samples of the water-soluble and lipid-soluble extracts from the cells and medium were taken for determination of ^{3}H by liquid-scintillation counting. In addition, samples of the cellular and medium water-soluble extracts were analysed by t.l.c. on plates of silica gel G. A sample (10μl) of carrier mixture that contained choline (30mg/ml), phosphocholine (60mg/ml) and betaine (30mg/ml) was also applied to each lane and the plates were developed in methanol/0.6% NaCl/NH_3 (10:10:1, by vol.). Spots were made visible with I_2 vapour and areas of the plates were scraped into counting vials and assayed for radioactivity.

Measurement of cellular phosphocholine

Phosphocholine concentrations were measured in the water-soluble extract of the cells by the method of Choy *et al.* (1978). This assay relies on the conversion of phosphocholine and [^{3}H]CTP into [^{3}H]CDP-choline by partially purified CTP:phosphocholine cytidylyltransferase. The assay was sensitive in the 0–1nmol range with a higher specific radioactivity of [^{3}H]CTP than previously reported. The assay was complicated by the presence of ^{3}H from [^{3}H]phosphocholine in the sample as well as the radioactivity from the [^{3}H]CTP. This was accounted for by incubating control reactions for each sample in which non-radioactive CTP was used in place of [^{3}H]CTP.

Results

Yield and viability of cells

Approx. 1×10^8 hepatocytes were recovered from each rat liver and 85–90% of these cells excluded 0.04% Trypan Blue. Adhesion rates were usually about 80%. After the 24h plating period, the viable cells had lost their round granular appearance and had begun to flatten. At this time only70–80% of the plated cells excluded the dye but non-viable cells were removed by careful washing of the dishes with culture medium. Cell viability at 24h was also assessed by their ability to secrete many serum

proteins (including albumin, transferrin, apo AI, apo E, anti-trypsin and fibrinogen) into the culture medium (P. H. Pritchard & D. E. Vance, unpublished work). The average wet weight of the cells that remained in each dish after washing was 10mg. This was determined by weighing the cells recovered from 10 plates after incubation in a trypsin- and EDTA-containing medium.

Metabolism of [Me-^{3}H]choline by hepatocytes

The cultured hepatocytes efficiently incorporated and metabolized the labelled choline from the medium during the 15min pulse period. Choline transported into the cells was rapidly oxidized to betaine or phosphorylated to phosphocholine (Fig. 1). Only about 6% of the incorporated label was associated with free cellular choline at the end of the pulse period and only about 1% of the incorporated label had accumulated in phosphatidylcholine at this time. The rapid oxidation of choline to betaine by cultured hepatocytes is similar to that observed in rat liver *in vivo* (Wong & Thompson, 1972; Sundler *et al.*, 1972; P. H. Lim, P. H. Pritchard & D. E. Vance, unpublished work), rat liver slices (Weinhold & Sanders, 1973) and by freshly isolated hepatocytes (Sundler & Åkesson, 1975).

Fig. 2 shows the effect of choline concentration in the medium on the relative incorporation of [*Me*-^{3}H]choline into choline, phosphocholine and betaine of the hepatocytes. The relative accumulation of choline from the medium into cellular choline was unaffected by the medium concentration. However, as the medium concentration of choline was increased from 1μM to 40μM the relative incorporation of [*Me*-^{3}H]choline into phosphocholine decreased from 58$\pm$3 to 33$\pm$1%, whereas that oxidized to betaine increased from 35$\pm$2 to 57$\pm$2% (Fig. 2). The concentration of choline had no effect on the relative accumulation of label in phosphatidylcholine during the 15min pulse period. The results indicate that the concentration of choline in

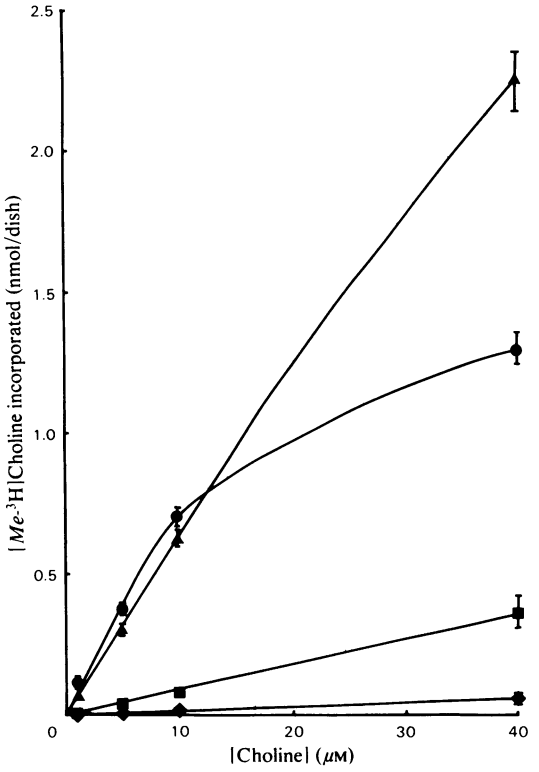

Fig. 1. *The effect of choline concentration on the incorporation and metabolism of [Me-^{3}H]choline by cultured hepatocytes*

Hepatocytes cultured for 24h (approx. 10mg of cells/dish) were incubated for 15min in medium containing 20μCi of [*Me*-^{3}H]choline and different concentrations of unlabelled choline. The incorporation of label into betaine (▲), phosphocholine (●), cellular choline (■) and phosphatidylcholine (♦) was determined by t.l.c. of the water- and lipid-soluble extracts of the cells. Data points are means $\pm$ S.E.M. for four to six incubations from two or three preparations of hepatocytes.

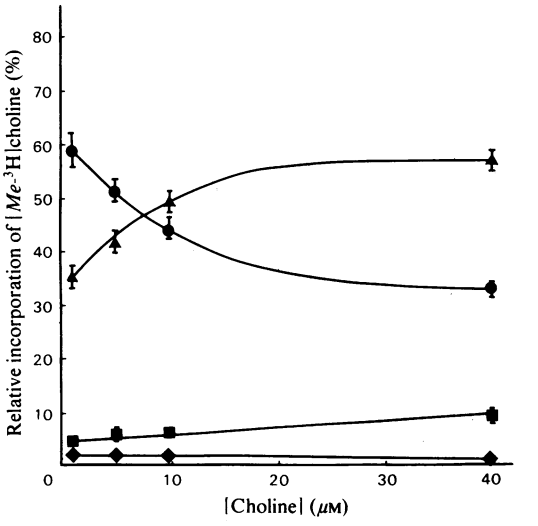

Fig. 2. *The effect of choline concentration on the relative extent of choline oxidation or phosphorylation by cultured hepatocytes*

Hepatocytes were incubated with [*Me*-^{3}H]choline as described for Fig. 1. The relative extent of choline oxidation or phosphorylation is shown by the percentage of incorporated label recovered in betaine (▲) or phosphocholine (●) respectively. The percentage of label recovered as cellular choline or phosphatidylcholine is shown by ■ or ♦ respectively.

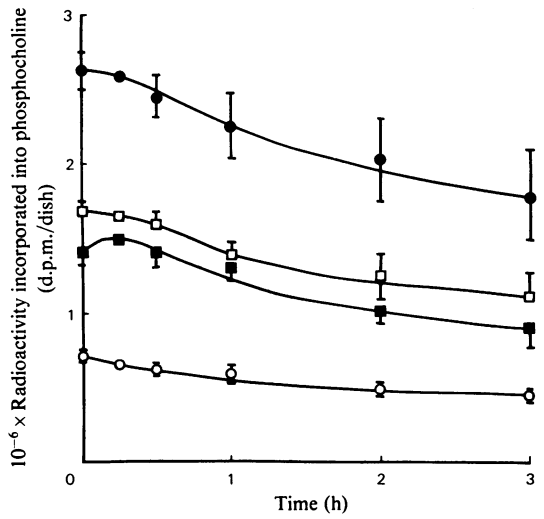

Fig. 3. *The loss of label from the phosphocholine pool after incubation with [Me-^{3}H]choline*
Hepatocytes were incubated for 15 min with [*Me*-^{3}H]choline as described for Fig. 1. The cells were washed free of label and then incubated with a non-radioactive chase medium for up to 3 h (see the Materials and methods section). The Figure shows the loss of label from phosphocholine in cells incubated in 1 μM- (●), 5 μM- (□), 10 μM- (■) and 40 μM- (○) choline. Each point is the mean ± S.E.M. for four or six incubations from two or three preparations of hepatocytes.

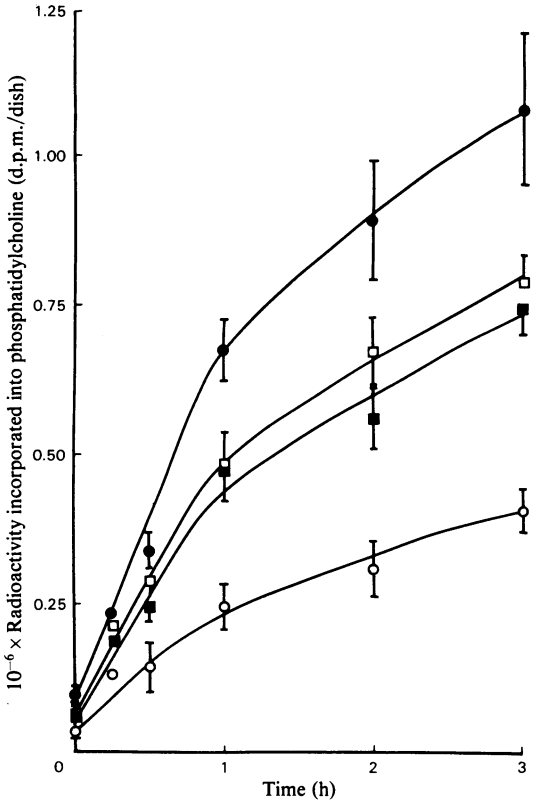

Fig. 4. *The accumulation of label into phosphatidylcholine after incubation with [Me-^{3}H]choline*
Hepatocytes were pre-incubated with [*Me*-^{3}H]-choline and then incubated in a chase medium as described for Fig. 3. Label recovered in phosphatidylcholine from cells incubated in 1, 5, 10 and 40 μM-choline is shown in ●, □, ■ and ○ respectively. Each point is the mean ± S.E.M. for four or six incubations from two or three preparations of hepatocytes.

the medium influences the proportion of choline that is oxidized or phosphorylated.

The metabolism of the label incorporated into betaine and phosphocholine was studied by replacing the labelled culture medium with an equivalent, but non-radioactive, medium. Figs. 3 and 4 show that, from 1 to 3 h of the chase period, radioactivity lost from the phosphocholine pool appears in phosphatidylcholine. Only negligible radioactivity (< 0.2%) was associated with the CDP-choline pool. Hence, the rate of loss of label from phosphocholine appears to govern the rate of accumulation of label in phosphatidylcholine. Since label does not accumulate in CDP-choline, the enzyme that converts phosphocholine into CDP-choline (the cytidylyltransferase) must catalyse the rate-limiting step for the incorporation of choline into phosphatidylcholine. Similar results were obtained at all concentrations of choline in the medium even though the amount of radioactivity associated with the phosphocholine pool at the start of the chase period was greatly decreased at higher choline concentrations (Fig. 3).

The major proportion of the radioactivity in the betaine pool at the end of the pulse period was recovered in the culture medium during the chase period (Fig. 5). However, a significant amount of the labelled betaine was not recovered in the medium. It is likely that this was transferred as methyl groups to homocysteine for the synthesis of methionine (Tyler, 1977). Similar results were obtained at other concentrations of choline (1, 5 and 40 μM).

Discussion

Choline concentration influences its metabolic fate in rat hepatocytes

The data in Fig. 2 clearly indicate that at choline concentrations below 10 μM, phosphorylation is favoured over oxidation. The choline is apparently conserved for phosphatidylcholine biosynthesis. This is consistent with the results of Wong & Thompson

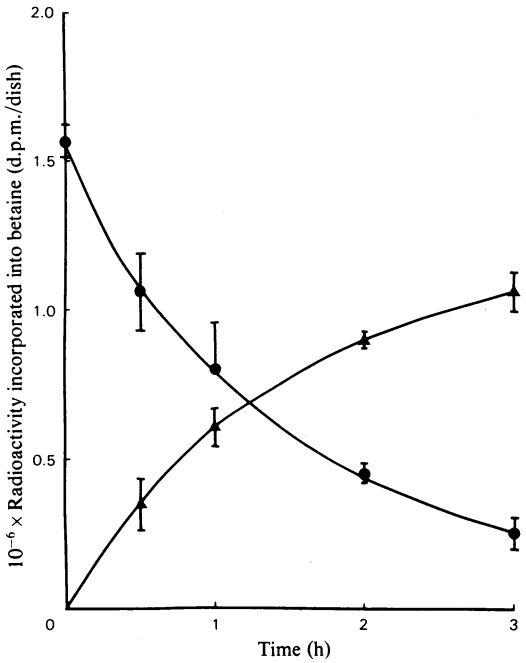

Fig. 5. *The secretion of labelled betaine into the culture medium after the incubation of hepatocytes with [Me-^{3}H]choline*

Hepatocytes were treated as described for Fig. 3 and the label recovered in cellular betaine and betaine in the culture medium is shown by ● and ▲ respectively. The Figure shows the results for cells incubated in 10μM-choline and data points are means ± S.E.M. Similar results were obtained with cells incubated at 1, 5 and 40μM-choline.

(1972), who reported that the concentration of betaine in rat liver was negligible after 2 days of choline deficiency. It is not known how the concentration of choline outside the cells modulates its metabolism inside the cell. There may be a competition between choline kinase and choline transport into the mitochondria (Tyler, 1977) where oxidation occurs. At low rates of choline entry the choline kinase reaction may predominate. The mechanism by which choline kinase could win this competition is not known. Recent studies on cockerels treated with diethylstilboestrol suggest that the activity of choline kinase in liver may determine the amount of choline directed towards phosphatidylcholine biosynthesis (C. Vigo & D. E. Vance, unpublished work). A higher activity for choline kinase *in vivo* and *in vitro* correlated with a diversion of choline from betaine to phosphocholine. Whether or not that is true in rat hepatocytes remains to be established. This question might be addressed in studies with hepatocytes derived from essential-fatty-acid-deficient rats. It has been reported that these rats have over a 3-fold increase in choline kinase when assayed *in vitro* (Infante & Kinsella, 1978).

We were impressed by the large percentage of cellular betaine that was released into the medium by the hepatocytes. Weinhold & Sanders (1973) reported similar results with rat liver slices. It is well established that betaine can serve as a methyl donor in liver for the synthesis of methionine from homocysteine (Tyler, 1977). Perhaps betaine produced in liver also serves as a methyl donor for methionine synthesis in non-hepatic tissues.

A rate-limiting role in phosphatidylcholine biosynthesis for CTP:phosphocholine cytidylyltransferase

The evidence from a number of studies favours the conclusion that the activity of the cytidylyltransferase determines the rate of phosphatidylcholine biosynthesis (Vance & Choy, 1979). The best evidence has been obtained with HeLa cells infected with polio virus (Vance *et al.*, 1980). Labelled choline that enters these cells is rapidly phosphorylated. As the radioactivity disappears from the phosphocholine, it appears in phosphatidylcholine. Polio virus stimulates to the same degree both the disappearance of label from phosphocholine and synthesis of phosphatidylcholine. The amount of radioactivity in CDP-choline is always very low, although the specific radioactivity approaches the same value as obtained with phosphocholine.

Until the present work, the case for a rate-limiting role of the cytidylyltransferase in rat liver has been circumstantial. The two main points in support of this hypothesis were the high ratio (150) of phosphocholine to CDP-choline in liver (Sundler *et al.*, 1972) and the incorporation studies in hepatocytes of choline into phosphocholine and phosphatidylcholine (Sundler & Åkesson, 1975). The results in Figs. 3 and 4 clearly indicate that as the label disappears from phosphocholine it appears in phosphatidylcholine. In the light of these results it is hard to imagine how any other reaction of phosphatidylcholine biosynthesis in hepatocytes could be the slow step. Support for this conclusion has been obtained in rats fed a diet rich in cholesterol and cholic acid. These rats showed a 2–3-fold elevation in the cytidylyltransferase activity with no effect on choline kinase or CDP-choline:1,2-diacylglycerol phosphocholinetransferase. At the same time the rate of phosphatidylcholine biosynthesis was stimulated 3-fold *in vivo* (P. H. Lim, P. H. Pritchard & D. E. Vance, unpublished work).

The mechanisms by which the activity of the cytidylyltransferase is governed remain to be established. Three mechanisms have been proposed and supported by experimental studies. In HeLa cells,

Table 1. *The effect of medium choline concentration on the rate of phosphatidylcholine biosynthesis in cultured hepatocytes*
The rate of phosphatidylcholine biosynthesis was calculated for a period of 1–3 h after the start of the chase incubation. The radioactivity in the phosphocholine pool at the start of each 0.5 h interval was determined from Fig. 3. The accumulation of label into phosphatidylcholine during each 0.5 h period was determined from Fig. 4. Phosphocholine concentrations of cells incubated at different choline concentrations were not significantly different and so the mean (± S.D.) value of 13.4 ± 2.5 nmol/dish (n = 38) was used to calculate its specific radioactivity. Each dish contained approx. 2.5 × 10^6 hepatocytes with an average wet weight of 10 mg.

Medium choline concn. (μM)	Chase period (h)	10^{-3} × ^{3}H in phosphocholine (d.p.m./dish)	10^{-3} × Specific radioactivity of phosphocholine (d.p.m./nmol)	10^{-3} × Accumulation of ^{3}H in phosphatidylcholine (d.p.m./0.5 h per dish)	Rate of phosphatidylcholine biosynthesis (pmol/0.5 h per dish)	
1	1.0–1.5	2250	168	126	750	660 ± 30
	1.5–2.0	2080	155	98	632	
	2.0–2.5	1960	146	93	640	
	2.5–3.0	1850	138	85	616	
5	1.0–1.5	1400	104	90	865	825 ± 18
	1.5–2.0	1280	96	80	833	
	2.0–2.5	1200	90	70	778	
	2.5–3.0	1140	85	70	824	
10	1.0–1.5	1240	93	85	914	926 ± 6
	1.5–2.0	1090	81	75	926	
	2.0–2.5	1000	75	70	933	
	2.5–3.0	930	69	65	942	
40	1.0–1.5	560	42	50	1190	1121 ± 34
	1.5–2.0	520	39	45	1154	
	2.0–2.5	480	36	40	1111	
	2.5–3.0	450	34	35	1029	

the activity of this enzyme can apparently be regulated by the concentration of the substrate, CTP, in the cytoplasm (Vance *et al.*, 1980; Choy *et al.*, 1980). In rat liver the activity of the cytidylyltransferase can be modulated by the concentration of a lipid, lysophosphatidylethanolamine (Choy & Vance, 1978; Vance & Choy, 1979). Similarly, phosphatidylglycerol may modulate the activity of the cytidylyltransferase in lung (Feldman *et al.*, 1978; Rooney, 1979). Finally, in rooster liver, it appears that the concentration of phosphocholine can alter the rate of this reaction (C. Vigo & D. E. Vance, unpublished work).

The effect of choline concentration on the rate of phosphatidylcholine biosynthesis

From the pulse–chase studies we have calculated the rate of phosphatidylcholine biosynthesis in cultured hepatocytes. This was determined during the period 1–3 h of the chase incubation (Table 1). The specific radioactivity of the phosphocholine pool at different times was determined from the data in Fig. 3 and from the total amount of phosphocholine present in the cells (Table 1). The concentration of phosphocholine in the cells was independent of the concentration of choline in the medium. The accumulation of label into phosphatidylcholine during a 0.5 h period was determined from Fig. 4. Hence, the rate of phosphatidylcholine biosynthesis could be determined by dividing the rate of accumulation of label into phosphatidylcholine by the specific radioactivity of phosphocholine at the start of the time interval studied (Table 1). For this calculation we assumed that the concentrations of CDP-choline were low in the hepatocyte and that the turnover-time of CDP-choline was very rapid compared with phosphocholine. This assumption seems valid because the label that disappears from phosphocholine immediately accumulates in phosphatidylcholine (Figs. 3 and 4) with very low radioactivity associated with CDP-choline.

From Table 1 we can see that the rate of phosphatidylcholine biosynthesis gradually increases between 1 and 40 μM-choline in the medium. This change in rate was not accompanied by an alteration in the concentration of phosphocholine. It is not clear to us how the rate of phosphatidylcholine biosynthesis can vary with choline concentration in the medium without major changes in the phosphocholine pool.

Each dish contained about 10 mg of cells. Hence the concentration of phosphocholine in these hepatocytes was 1.3 μmol/g of cells. This is similar to the value of 1.1 μmol/g wet weight reported for rat liver (Choy *et al.*, 1978) and well above the apparent K_m of 0.17 mM for this substrate by the cytidylyltransferase (Choy *et al.*, 1978).

If we again use the value of 10mg of cells per dish and the data in Table 1, we can estimate the rate of phosphatidylcholine biosynthesis per g of cells. This calculation gives values of 2.2, 2.8, 3.1 and 3.7nmol/min per g wet weight of cells cultured in 1, 5, 10 and 40μM-choline respectively. These rates are lower than those reported for rat liver *in vivo* (20nmol/min per g of liver) (Sundler *et al.*, 1972). This could be due to a decrease in the activity of the cytidylyltransferase, which is reduced 4-fold in 24h-cultured hepatocytes (S. Pelech, P. H. Pritchard & D. E. Vance, unpublished work). A decrease in enzyme activities in cultured hepatocytes has been observed with several other enzymes. For example, the activity of glucokinase decreases by 90% during the first 8h of incubation and remains constant thereafter (Spence & Pitot, 1979). Similar results were reported for stearoyl-CoA desaturase when insulin was absent from the culture medium (Jeffcoat *et al.*, 1979).

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