The choline-depleted type II pneumonocyte

A model for investigating the synthesis of surfactant lipids

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(Received 9 July 1984/Accepted 23 July 1984)

When type II pneumonocytes from adult rats were maintained in a medium that lacked choline, the incorporation of [^{14}C]glycerol into phosphatidylcholine was not greatly diminished during the period that the cells displayed characteristics of type II pneumonocytes. Cells that were maintained in choline-free medium that contained choline oxidase and catalase, however, became depleted of choline and subsequent synthesis of phosphatidylcholine by these cells was responsive to choline in the extracellular medium. Incorporation of [^{14}C]glycerol into phosphatidylcholine by choline-depleted cells was stimulated maximally (approx. 6-fold) by extracellular choline at a concentration (0.05 mM) that also supported the greatest incorporation into phosphatidylglycerol. The incorporation of [^{14}C]glycerol into other glycerophospholipids by choline-depleted cells was not increased by extracellular choline. When cells were incubated in the presence of [^3H]cytidine, the choline-dependent stimulation of the synthesis of phosphatidylcholine and phosphatidylglycerol was accompanied by an increased recovery of [^3H]CMP. This increased recovery of [^3H]CMP reflected an increase in the intracellular amount of CMP from 48 ± 9 to 76 ± 16 pmol/ 10^6 cells. Choline-depleted cells that were exposed to [^3H]choline contained [^3H]CDP-choline as the principal water-soluble choline derivative. As the extracellular concentration of choline was increased, however, the amount of ^3H in phosphocholine greatly exceeded that in all other water-soluble derivatives. Choline-depletion of cells resulted in an increase in the specific activity of CTP:phosphocholine cytidyltransferase in cell homogenates (from 0.40 ± 0.15 to 1.31 ± 0.20 nmol $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$). These data are indicative that the biosynthesis of phosphatidylcholine is integrated with that of phosphatidylglycerol and are consistent with the proposed involvement of CMP in this integration. The choline-depleted type II pneumonocyte provides a new model for investigating the regulation of CTP:phosphocholine cytidyltransferase activity.

The most abundant glycerophospholipids of the surfactant that is produced by type II pneumonocytes of mature lungs are phosphatidylcholine and phosphatidylglycerol (Body, 1971; Pfleger & Thomas, 1971). The surfactant produced by immature lungs, however, contains phosphatidylinositol in place of phosphatidylglycerol (Hallman & Gluck, 1975; Hallman *et al.*, 1977). Foetal lung

maturation in several species is accompanied by a developmental decline in the amount of phosphatidylinositol in lung surfactant that is concomitant with an increase in the phosphatidylglycerol content of surfactant (Hallman & Gluck, 1980). There is indirect evidence that phosphatidylglycerol may be essential for proper surfactant function (Hallman *et al.*, 1976) but the role of phosphatidylglycerol in surfactant remains unknown (Beppu *et al.*, 1983). The mechanisms that operate within the type II pneumonocyte to regulate the

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biosynthesis of phosphatidylglycerol and phosphatidylinositol from their common precursor, CDP-diacylglycerol, are not well understood. There is evidence, however, that CDP-diacylglycerol:inositol 3-phosphatidyltransferase (EC 2.7.8.11, the enzyme that catalyses the final reaction in phosphatidylinositol biosynthesis) is involved in the regulation of phosphatidylglycerol biosynthesis (Bleasdale *et al.*, 1979; Quirk *et al.*, 1980; Bleasdale & Johnston, 1982a). First, activity of this enzyme in type II pneumonocytes is sensitive to developmental changes in the availability of *myo*-inositol; when the concentration of *myo*-inositol in foetal serum decreases in late gestation, CDP-diacylglycerol:inositol 3-phosphatidyltransferase activity decreases and more CDP-diacylglycerol is available for phosphatidylglycerol biosynthesis (Bleasdale *et al.*, 1982). Second, CDP-diacylglycerol:inositol 3-phosphatidyltransferase catalyses a reversible reaction and the reverse reaction is promoted by CMP (Bleasdale *et al.*, 1979). Intracellular amounts of CMP in foetal lung tissue increase in late gestation as a result of enhanced phosphatidylcholine synthesis for surfactant (Quirk *et al.*, 1980). Consequently, the net biosynthesis of phosphatidylinositol decreases and more CDP-diacylglycerol is available for phosphatidylglycerol biosynthesis. The objective of this investigation was to alter the rate of phosphatidylcholine biosynthesis by type II pneumonocytes and to examine the effect on the rate of biosynthesis of phosphatidylglycerol. Since the rate of phosphatidylcholine synthesis by type II pneumonocytes *in vitro* can be increased relatively little by hormones (G. C. Di Renzo & J. E. Bleasdale, unpublished work; Post *et al.*, 1980) we sought to alter phosphatidylcholine biosynthesis greatly by manipulating the availability of choline.

Materials and methods

Materials

Male Sprague-Dawley rats (200–225 g), free from specific pathogens, were obtained from Blue Spruce Farms Inc., Altamont, NY, U.S.A., and were housed inside a laminar-flow hood and fed *ad libitum* until killed. Choline oxidase purified from *Arthrobacter globiformis* (specific activity 12 μmol of H_2O_2 formed $\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$, pH 8.0, 25°C) was obtained from Tokyo Jozo Co., Tokyo, Japan, and catalase purified from bovine liver (specific activity 40 mmol of H_2O_2 consumed $\cdot\text{min}^{-1}\cdot\text{mg}$ of protein $^{-1}$, pH 7.0, 25°C) was obtained from Worthington Biochemical Corporation, Freehold, NJ, U.S.A. 2-Hydroxy-3,5-dichlorobenzenesulphonate (sodium salt) was from Research Organics Inc., Cleveland, OH, U.S.A. Dulbecco's modified Eagle's medium

(with or without choline) was prepared by using chemicals obtained from Sigma Chemical Co. The sources of all other materials have been described previously (Bleasdale *et al.*, 1982, 1983). Type II pneumonocytes were isolated by a modification of the method of Mason *et al.* (1977a,b) as described elsewhere (Bleasdale *et al.* 1982).

Choline-depletion and incubation of cells

Isolated cells were washed once by suspending in Dulbecco's modified Eagle's medium that contained antibiotics and an antimycotic (Bleasdale *et al.*, 1982) (referred to subsequently as medium) followed by centrifugation at 250g for 6 min. The washed cell pellet was resuspended in medium. Cells (approx. 2×10^6 in 1 ml of medium) were incubated in glass screw-cap culture tubes (150 mm $\times$ 20 mm) in an incubator at 37°C. [^{14}C]Glycerol (0.07 mM, approx. 30 Ci/mol) was added to the cell suspension and incubation was continued for 12 h unless stated otherwise. For most experiments, however, incubation of the cells with [^{14}C]glycerol was preceded by treatment of the cells with choline oxidase and catalase as follows. The washed cell pellet (consisting of approx. 5×10^7 cells) was resuspended in 10 ml of choline-free medium that contained choline oxidase (1.3 mg of protein) and catalase (0.08 mg of protein) and the mixture was incubated at 37°C for 3 h. The cells were then collected by centrifugation (250g for 6 min) and washed twice in choline-free medium before being resuspended in medium that contained [^{14}C]glycerol (0.07 mM, approx. 30 Ci/mol) and choline at various concentrations. Cells were then incubated as described above. After incubation, 4.5 ml of chloroform/methanol (1:2, v/v) and 0.1 ml of HCl (6M) were added to each tube and lipids were extracted (Bleasdale *et al.*, 1979). Glycerophospholipids in the total lipid extract were separated by using the two-dimensional t.l.c. system of Yavin & Zutra (1977) and neutral lipids were separated on silica gel H plates developed in heptane/diethyl ether/acetic acid (75:25:4, by vol.). Incorporation of [^{14}C]glycerol into individual lipid classes was measured as described previously (Bleasdale *et al.*, 1983).

Analysis of cytidine nucleotides

In some experiments, cells were incubated in the presence of [^3H]cytidine (60 μM , 135 Ci/mol). After incubation, the cells were washed and radio-labelled cytidine nucleotides were extracted with 3% (v/v) HClO_4 (Quirk *et al.*, 1980) after adding to every sample CTP, CDP, CMP (0.1 mg of each) and cyclic CMP, CDP-choline and cytidine (0.2 mg of each). Extracts were neutralized (Khim, 1975) and then subjected to t.l.c. on 20 cm $\times$ 20 cm plates of poly(ethyleneimine)-cellulose (pre-

washed with water) which were developed sequentially in one dimension using 0.1M-LiCl (for the first 10cm of migration) and 1.4M-LiCl (for the remaining 8cm of migration). Cytidine nucleotides on chromatograms were located under u.v. light and areas corresponding to cytidine, CMP, CDP, CTP and CDP-choline were scraped into scintillation vials. Scintillation fluid (10ml) and 0.85ml of 3.5M-HCl were added to each vial and radioactivity was assayed (Bleasdale *et al.*, 1979). When intracellular CMP was quantified, nucleotides were extracted from cells with 3% (v/v) HClO₄ and the acid extracts were neutralized (as described above but omitting carrier nucleotides). CMP was separated and quantified using h.p.l.c. on a Whatman Partisil 10 SAX column (10µm particle size, 25cm×0.46cm) as described by Hartwick & Brown (1975). Data were corrected for the recovery of each nucleotide as described (Quirk *et al.*, 1980).

Analysis of choline and its derivatives

For the measurement of incorporation of ³H into intracellular choline, phosphocholine, CDP-choline and betaine, cells that had been incubated in medium which contained [³H]choline (at various concentrations) were collected by centrifugation (250g for 6min) and washed once with medium. Each cell pellet was resuspended in 1.0ml of methanol/water (5:4, v/v) and cells were disrupted with ultrasound for 30s (Bransonic 220 bath sonicator) (Post *et al.*, 1982). Choline (0.4mg), phosphocholine (1mg) and CDP-choline (2mg) were added to each sample together with chloroform (1.5ml), methanol (0.95ml) and water (0.75ml). After mixing well, samples were centrifuged (500g for 10min) to separate phases. Each upper phase was removed and evaporated under N₂. Each resulting residue was redissolved in water (0.1ml) and an aliquot (0.04ml) was subjected to t.l.c. for the separation of choline and its derivatives (Weinhold *et al.*, 1981). Areas of each chromatogram were scraped into scintillation vials and radioactivity was assayed (Bleasdale *et al.*, 1979). Identification of choline and its derivatives in cell extracts was confirmed by co-migration with authentic standards in a second chromatographic system. For this purpose ascending paper chromatography on Whatman no. 1 paper developed in butan-1-ol/water/1M-acetic acid/ethanol (3:3:1:2, by vol.) was performed (Haubrich *et al.*, 1974). When intracellular amounts of choline were measured, cells were disrupted by ultrasound (as described above) and then immersed in a boiling-water bath for 10min. The disrupted cells were centrifuged at 10000g for 5min and aliquots of the supernatant fluids were taken for the assay of choline as described by Muneshige *et al.* (1983),

except that phenol was replaced by 2-hydroxy-3,5-dichlorobenzenesulphonate (1.9mm) (McGowan *et al.*, 1983) and the concentration of 4-aminoantipyrine that was employed was 0.8mm.

Assay of CTP:phosphocholine cytidyltransferase activity

CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) activity was assayed employing a modification of the method described by Pelech *et al.* (1981). Cells were resuspended in Tris/succinate solution (30mm, pH6.5) that contained NaF (10mm) and EDTA (1mm) and were disrupted using ultrasound (as described above) followed by homogenization in a glass/glass hand-held homogenizer (12 strokes). Saline solution was added to each cell homogenate until the final concentration of NaCl was 154mm. The assay mixture consisted of Tris/succinate buffer (30mm, pH6.5), magnesium acetate (10mm), CTP (5mm), [¹⁴C]phosphocholine (1mm, 2.5Ci/mol) and cell homogenate (approx. 60µg of protein) in a total volume of 0.1ml. Incubations were continued for 20min at 37°C, and then were terminated by immersion in boiling water for 2min. Heat-denatured proteins were removed by centrifugation (2000g for 10min) and [¹⁴C]phosphocholine and [¹⁴C]CDP-choline in an aliquot (0.04ml) of the supernatant fluid were separated by t.l.c. (Weinhold *et al.*, 1981) after adding 0.01ml of CDP-choline (100mg/ml) as carrier. [¹⁴C]CDP-choline was quantified by liquid-scintillation spectrometry using a scintillation fluid that contained Triton X-100 (Bleasdale *et al.*, 1979). The rate of CTP:phosphocholine cytidyltransferase activity was proportional to the amount of protein employed (up to 100µg) and for a given amount of protein was constant for 20min.

Statistical significance between mean values was assessed by Student's *t*-test (Zar, 1974). Differences were considered significant at *P*<0.05. Other methods that were employed have been described previously (Bleasdale *et al.*, 1982).

Results

In a previous investigation, we measured the rate of incorporation of [¹⁴C]glycerol into phosphatidylcholine by type II pneumonocytes that were maintained in medium that contained choline (Bleasdale *et al.*, 1983). In the present investigation it was observed that the rate of incorporation of [¹⁴C]glycerol into phosphatidylcholine by untreated type II pneumonocytes was not greatly affected by withdrawal of choline from the medium (Fig. 1). Incorporation of [¹⁴C]glycerol into phosphatidylcholine over a 2h period was not affected significantly immediately following choline withdrawal and in a typical experiment was reduced by

Fig. 1. Effect of choline-withdrawal on phosphatidylcholine biosynthesis by untreated type II pneumonocytes

Rat type II pneumonocytes were suspended in Dulbecco's modified Eagle's medium with or without choline (0.03 mM) (2×10^6 cells/ml of medium) and were incubated at 37°C in an atmosphere of air/CO₂ (19:1). After various periods of incubation, the cells were exposed to [¹⁴C]glycerol (0.07 mM, approx. 30 Ci/mol) for 2 h. Cells were harvested, lipids were extracted and incorporation of ¹⁴C into phosphatidylcholine was measured (Methods). ▨, With choline; ■, without choline.

only 29–50% during the period 14–24 h after choline withdrawal. After longer periods of incubation, incorporation of [¹⁴C]glycerol into phosphatidylcholine declined markedly when cells were in either normal or choline-free medium (Fig. 1). Type II pneumonocytes in choline-free medium apparently contained sufficient choline to support normal biosynthesis of phosphatidylcholine in the short-term. For these reasons, we evaluated procedures for increasing the effect of choline on the incorporation of [¹⁴C]glycerol into phosphatidylcholine after relatively short periods of incubation.

It was found that type II pneumonocytes could be depleted of choline by treating the cells with choline-free medium that contained choline oxidase and catalase. The bacterial choline oxidase that was employed catalyses the two-step conversion of choline to betaine (Ikuta *et al.*, 1977). Catalase was included to protect the cells from H₂O₂ which is a co-product of the reactions catalysed by choline oxidase. When the amount of intracellular choline was quantified, it was found that exposure of cells to choline-free medium that contained choline oxidase resulted in a significant decrease in intracellular choline from 250 ± 75 to 84 ± 20 pmol/10⁶ cells (mean $\pm$ S.D., seven experiments). Assay sensitivity and the limited availability of cells precluded direct measurement of

intracellular CDP-choline. Therefore, to investigate the effect of the choline-depletion procedure on intracellular choline derivatives, type II pneumonocytes were incubated for either 3 h or 12 h in medium that contained [³H]choline (0.03 mM) and then were transferred to fresh medium that contained either non-radiolabelled choline (0.03 mM) or choline oxidase plus catalase. After 3 h in fresh medium, cells were separated from medium, washed twice and the amounts of ³H in intracellular choline, phosphocholine, CDP-choline and phosphatidylcholine and in extracellular choline, betaine and phosphatidylcholine were quantified (Table 1). In preliminary experiments (results not shown), it was found that exposure to [³H]choline for more than 5 h was required before intracellular water-soluble choline derivatives reached isotopic equilibrium. As summarized in Table 1, however, the effect of the choline-depletion procedure on the distribution of ³H among intracellular choline derivatives was qualitatively similar in cells that were exposed previously for either 3 h or 12 h to [³H]choline. The choline-depletion procedure did not affect the total recovery of intracellular and extracellular ³H, or the amount of ³H in intracellular CDP-choline and phosphatidylcholine. Recovery of ³H in intracellular choline and phosphocholine was diminished by the choline-depletion procedure. Some of the ³H

Table 1. *Loss of ^3H from choline and its derivatives in type II pneumonocytes that were exposed previously to [^3H]choline*

Type II pneumonocytes were incubated for either 3 h (experiment 1) or 12 h (experiment 2) at 37°C in Dulbecco's modified Eagle's medium that contained [^3H]choline (0.03 mM, approx. 30 Ci/mol). After the cells were incubated, they were washed twice in choline-free medium and then suspended either in choline-free medium that contained choline oxidase and catalase (depletion medium) or in medium that contained choline (0.03 mM) (normal medium). The cells were incubated for a further 3 h before being harvested, washed and subjected to the extraction and separation procedures for water-soluble derivatives of choline and for phosphatidylcholine (see the Materials and methods section). Choline, phosphocholine, CDP-choline and betaine together accounted for 83–90% of the ^3H in intracellular water-soluble compounds and this value was not affected by the choline-depletion procedure (range of eight determinations). Phosphatidylcholine accounted for 89–96% of ^3H in either intracellular or extracellular lipids. Choline and betaine together accounted for only 65–70% of ^3H in extracellular water-soluble compounds. The other ^3H -labelled water-soluble compounds recovered in the medium were not identified, but none accounted for more than 10% of the total ^3H in water-soluble compounds. The data are from two typical experiments. Abbreviation used: n.d., not determined.

	^3H recovered (d.p.m./ 10^6 cells)			
	Normal medium		Depletion medium	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Intracellular				
Choline	1864	4025	531	1803
Phosphocholine	9356	22117	3295	17417
CDP-choline	1129	4866	1124	4354
Betaine	n.d.	188	n.d.	171
Phosphatidylcholine	174286	1.24×10^6	173876	1.15×10^6
Extracellular				
Choline	9617	62471	2503	5614
Betaine	3046	1215	12373	54615
Phosphatidylcholine	n.d.	74918	n.d.	111361

taken up by the cells during the 3 h or 12 h exposure to [^3H]choline was subsequently released when the cells were transferred to fresh medium. Phosphatidylcholine, choline and betaine together accounted for 70–80% of the ^3H released from cells maintained for 3 h in either normal medium or choline-depletion medium. For cells in normal medium, however, extracellular [^3H]choline greatly exceeded [^3H]betaine, whereas for cells in choline-depletion medium there was an excess of extracellular [^3H]betaine. Intracellular [^3H]betaine was low and was unaffected by the choline-depletion procedure. These data, together with the direct measurements of intracellular choline (see above), support the view that choline inside type II pneumonocytes is exchangeable with extracellular choline. For cells in normal medium this process does not result in net loss of intracellular choline. When extracellular choline is replaced with choline oxidase and catalase, however, choline that is released from the cells is oxidized to betaine. This betaine is not taken up by the cells, which become depleted of choline (and probably phosphocholine also). The small amount of extracellular [^3H]betaine measured when cells were maintained in normal medium cannot be accounted for by contamination of the commercial [^3H]choline with [^3H]betaine and was apparently formed by the type II pneumonocytes (see also Figs. 4 and 5).

Phosphatidylcholine synthesis by choline-depleted type II pneumonocytes was now influenced by extracellular choline. When choline-depleted cells were incubated for 12 h in the presence of [^{14}C]glycerol, the incorporation of ^{14}C into phosphatidylcholine was increased approx. 6-fold when the concentration of choline in the medium was increased (Fig. 2). The incorporation of [^{14}C]glycerol into phosphatidylglycerol also was greatest at the concentration of choline (0.05 mM) that supported the greatest incorporation into phosphatidylcholine. The incorporation of ^{14}C into phosphatidylinositol was unaffected and the incorporation of ^{14}C into phosphatidylethanolamine decreased only 27% as the concentration of choline in the medium was increased (Fig. 3). With increasing concentration of extracellular choline, the incorporation of [^{14}C]glycerol into triacylglycerol decreased, that into diacylglycerol increased (Fig. 3) and that into monoacylglycerol was low and unchanged (results not shown). These observations are suggestive that when the extracellular concentration of choline is increased, some diacylglycerol is diverted away from triacylglycerol biosynthesis toward phosphatidylcholine, but that phosphatidylethanolamine biosynthesis is largely spared. Although the specific radioactivities of intracellular glycerol and glycerol 3-phosphate were not measured, it

Fig. 2. Influence of extracellular choline on glycerolipid synthesis by choline-depleted type II pneumonocytes. Type II pneumonocytes were depleted of choline for 3 h (see the Materials and methods section) and then were washed twice. Choline-depleted cells were suspended in medium that contained [^{14}C]glycerol (0.07 mM, approx. 30 Ci/mol) and choline at various concentrations. After 12 h of incubation at 37°C, cells were harvested and the incorporation of ^{14}C into glycerolipids was measured. ●, Phosphatidylcholine; ○, phosphatidylglycerol; ▲, phosphatidylinositol.

Fig. 3. Effect of choline on glycerolipid synthesis by choline-depleted type II pneumonocytes.

Choline-depleted type II pneumonocytes were exposed to [^{14}C]glycerol (0.07 mM, approx. 30 Ci/mol) and choline in various concentrations. After 12 h of incubation at 37°C, cells were harvested and the incorporation of ^{14}C into glycerolipids was measured. ●, Triacylglycerol; ○, diacylglycerol; ▲, phosphatidylethanolamine.

is likely that the choline-dependent changes in incorporation of [^{14}C]glycerol into glycerolipids reflect changes in amounts of lipid synthesized and not changes in specific radioactivities of precursors. This is because choline-dependent alterations in [^{14}C]glycerol incorporation into phosphatidylinositol (or any glycerolipid other than those mentioned above) were not observed.

The influence of extracellular choline on the intracellular relative amounts of choline and its derivatives was also investigated. Type II pneumonocytes were pretreated with choline-free medium that contained choline oxidase and catalase. Cells were washed twice with choline-free medium and then were transferred to fresh medium that contained [^3H]choline at various concentrations. At all concentrations of extracellular choline examined, incorporation of ^3H into intracellular choline, phosphocholine, CDP-choline and betaine increased for approx. 5 h and then remained unchanged for at least 12 h (results not shown). Presumably, between 5 h and 12 h the relative distribution of ^3H among different choline derivatives reflects the relative intracellular amounts of these compounds. Intracellular betaine appears to originate within the type II pneumonocytes, since cells not exposed to bacterial choline

oxidase also produce betaine and, in addition, when betaine is generated extracellularly by bacterial choline oxidase it is not taken up by the cells (Table 1). The distribution of intracellular ^3H among choline and its derivatives was found to be dependent upon the extracellular concentration of choline (Figs. 4 and 5). When exposed to low concentrations of [^3H]choline (0.04 μM), choline-depleted cells contained CDP-[^3H]choline as the most highly radiolabelled choline derivative (Fig. 4). At higher concentrations of extracellular [^3H]choline, however, [^3H]phosphocholine was by far the most highly radiolabelled choline derivative (Fig. 5). These data are consistent with previous reports that in many tissues the formation of CDP-choline (catalysed by CTP:phosphocholine cytidyltransferase) is a rate-limiting reaction in the incorporation of choline into phosphatidylcholine (see Vance & Choy, 1979, for a review). For this reason, the possibility that the specific activity of CTP:phosphocholine cytidyltransferase may respond to changes in the extracellular concentration of choline was also examined. Type II pneumonocytes were depleted of choline for 3 h and then transferred to medium that either lacked or contained choline (0.05 mM) and were incubated for 12 h. Type II pneumonocytes were then harvested by centrifugation (250g for 10 min), washed once, and then disrupted as described in the Materials and methods section. CTP:phosphocholine cytidyltransferase activi-

Fig. 4. Distribution of intracellular ^3H among choline, betaine, phosphocholine and CDP-choline

Choline-depleted type II pneumonocytes were exposed to [^3H]choline ($0.04\ \mu\text{M}$) and after 12 h at 37°C the cells were harvested and washed. [^3H]Choline and [^3H]choline derivatives were extracted from cells, separated and quantified (see the Materials and methods section).

Fig. 5. Distribution of intracellular ^3H among choline, betaine, phosphocholine and CDP-choline

Choline-depleted type II pneumonocytes were exposed to [^3H]choline at various concentrations and after 12 h at 37°C the cells were harvested and washed. [^3H]Choline and [^3H]choline derivatives were extracted from cells, separated and quantified (see the Materials and methods section). (a) $0.01\ \text{mM}$ -choline; (b) $0.02\ \text{mM}$ -choline; (c) $0.05\ \text{mM}$ -choline.

ties in the cell homogenates were measured. Specific activity of CTP:phosphocholine cytidyltransferase in homogenates of type II pneumonocytes that were maintained for 12 h in choline-free medium ($1.31 \pm 0.20\ \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) was significantly greater than that of homogenates of cells exposed to choline ($0.05\ \text{mM}$) ($0.40 \pm 0.15\ \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) (mean $\pm$ S.D., five experiments). The lower measured activity of CTP:phosphocholine cytidyltransferase in cells exposed to choline was not due to an increased amount of phosphocholine in such cells leading to a decrease in the specific radioactivity of the [^3H]phosphocholine that was employed as substrate in the enzyme assay. In a typical experiment, the activities of CTP:phosphocholine cytidyltransferase in homogenates of normal cells, of choline-depleted cells and of a mixture of equal amounts (mg of protein) of each homogenate were 0.43, 1.25 and $0.86\ \text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ respectively.

We have previously presented evidence that CMP may be involved in the integration of the biosynthesis of phosphatidylglycerol with that of phosphatidylcholine (Bleasdale & Johnston, 1982a,b; Quirk *et al.*, 1980). This proposition was tested further by incubating type II pneumonocytes in medium that contained [^3H]cytidine. Incorporation of [^3H]cytidine into various cytidine nucleotides increased for approximately 8 h and

then remained unchanged (results not shown). After 8 h the relative distribution of ^3H among various cytidine nucleotides presumably reflects the relative amounts of these nucleotides. When type II pneumonocytes were incubated for 12 h in the presence of [^3H]cytidine, the amount of ^3H recovered in CMP (and CTP) increased as the concentration of choline in the medium was increased. The amount of ^3H recovered in CDP-choline decreased, while that recovered in CDP remained unchanged (Fig. 6). The apparent increase in intracellular CMP as a result of increas-

Fig. 6. Effect of choline on the incorporation of [^3H]cytidine into various cytidine nucleotides by type II pneumonocytes

Choline-depleted type II pneumonocytes were suspended in medium that contained [^3H]cytidine (0.06 mM, 135 Ci/mol) and choline at various concentrations. After the cells were incubated at 37°C for 12 h, nucleotides were extracted and separated (see the Materials and methods section). Incorporation of ^3H into CMP (●), CDP (□), CTP (▲) and CDP-choline (○) was measured. Data are mean values derived from two experiments (range for each value <15% of mean).

ing the extracellular concentration of choline was confirmed in experiments in which choline-depleted type II pneumonocytes were incubated for 12 h in either choline-free medium or in medium that contained choline (0.05 mM) and after incubation, the intracellular amount of CMP was quantified by h.p.l.c. Type II pneumonocytes maintained in choline-free medium contained 48 ± 9 pmol of CMP/ 10^6 cells, while type II pneumonocytes exposed to choline (0.05 mM) contained 76 ± 16 pmol of CMP/ 10^6 cells (mean $\pm$ S.D., four determinations, $P < 0.05$).

Discussion

The objective of this investigation was to examine the integration between the biosynthesis of phosphatidylcholine and that of phosphatidylglycerol in type II pneumonocytes. As a model of the increase in phosphatidylcholine biosynthesis that occurs in foetal lungs during late gestation, phosphatidylcholine biosynthesis by type II pneumonocytes *in vitro* was altered by manipulating the

availability of choline. Type II pneumonocytes contained sufficient choline to support normal synthesis of phosphatidylcholine for several hours even when the cells were in choline-free medium. Although the rate of phosphatidylcholine synthesis by choline-starved cells did eventually decline, this was not related to the lack of choline but to the sub-optimal conditions of culture and was observed also with cells in medium that contained choline (Mason & Dobbs, 1980; Bleasdale *et al.*, 1983). Batenburg *et al.* (1982) also reported that when type II pneumonocytes were maintained in balanced salt solution, synthesis of phosphatidylcholine was relatively insensitive to the amount of choline in the solution. We observed, however, that under appropriate conditions type II pneumonocytes can be depleted of choline and that phosphatidylcholine biosynthesis by these cells is now extremely responsive to choline in the extracellular medium. The mechanism by which type II pneumonocytes were depleted of choline is not known but the data are indicative that the net efflux of choline from type II pneumonocytes is increased when the cells are incubated in medium that lacks choline but contains choline oxidase.

When choline-depleted type II pneumonocytes were incubated in medium that contained [^3H]choline at various concentrations, the distribution of ^3H among intracellular choline and its derivatives was consistent with the view that at low concentrations of extracellular choline the rates of formation of phosphocholine, CDP-choline and phosphatidylcholine are all submaximal. At higher concentrations of extracellular choline, the rate of formation of phosphocholine increases greatly and exceeds the rate of formation of CDP-choline. These findings are in agreement with previous evidence that CTP:phosphocholine cytidyltransferase catalyses a rate-limiting reaction in the incorporation of choline into phosphatidylcholine in lung tissue and may be a regulatory enzyme (Feldman *et al.*, 1981; Weinhold *et al.*, 1981). Choline-depletion of type II pneumonocytes resulted in an activation of CTP:phosphocholine cytidyltransferase. Characteristics of this activation are described elsewhere (Anceschi & Bleasdale, 1984). The mechanism of activation of CTP:phosphocholine cytidyltransferase is currently under investigation; however, mechanisms by which the activity of this enzyme may be controlled in other tissues have been described (e.g., Feldman *et al.*, 1978; Pelech *et al.*, 1983; Sleight & Kent, 1983). It should be noted that the increase in CTP:phosphocholine cytidyltransferase activity that was observed in choline-depleted type II pneumonocytes was rapid and so is likely to be unrelated to the slow decrease in activity of this enzyme that was observed in the

livers of rats that were fed a choline-deficient diet for more than 48 h (Schneider & Vance, 1978).

In type II pneumonocytes, the stimulation of phosphatidylcholine synthesis by choline was associated with an apparent diversion of some diacylglycerol away from triacylglycerol synthesis while phosphatidylethanolamine synthesis was largely spared. Furthermore, stimulation of phosphatidylcholine synthesis was accompanied by increased total utilization of CDP-diacylglycerol and a redirection of CDP-diacylglycerol in favour of phosphatidylglycerol synthesis. The extracellular concentration of choline that supported the greatest incorporation of [^{14}C]glycerol into phosphatidylcholine by choline-depleted type II pneumonocytes was that which also supported greatest incorporation of [^{14}C]glycerol into phosphatidylglycerol and resulted in the greatest recovery of [^3H]cytidine in CMP. The increased recovery of [^3H]CMP was found to reflect an increase in the intracellular concentration of CMP and this is consistent with our proposition that CMP is involved in the integration of the biosynthesis of phosphatidylcholine and that of phosphatidylglycerol (Bleasdale & Johnston, 1982b). In this integration, it was envisioned that the reversible reaction that is catalysed by CDP-diacylglycerol:inositol 3-phosphatidyltransferase is near equilibrium *in vivo* and is responsive to changes in the intracellular concentrations of CMP and *myo*-inositol. CMP is a product of the reaction catalysed by cholinephosphotransferase and the intracellular concentrations of CMP increase at a time in foetal lung development when the biosynthesis of phosphatidylcholine for surfactant is stimulated (Quirk *et al.*, 1980). By promoting the reverse reaction catalysed by CDP-diacylglycerol:inositol 3-phosphatidyltransferase, CMP inhibits the biosynthesis of phosphatidylinositol (relative to that of phosphatidylglycerol) and increases the availability of CDP-diacylglycerol (synthesized from phosphatidic acid) for the biosynthesis of phosphatidylglycerol. Similarly, a decline in the availability of *myo*-inositol for the foetal lungs that occurs in late gestation (Bleasdale *et al.*, 1982) results in an increase in the synthesis of phosphatidylglycerol at the expense of phosphatidylinositol biosynthesis (Hallman & Gluck, 1980; Bleasdale *et al.*, 1983). Thus the developmental decline in *myo*-inositol availability complements the developmental increase in the intracellular concentration of CMP and both promote phosphatidylglycerol biosynthesis by type II pneumonocytes [and do not act antagonistically as proposed by Batenburg *et al.* (1982)].

The results of this investigation are indicative that the choline-depleted type II pneumonocyte may be a suitable experimental model for

examining the regulation of CTP:phosphocholine cytidyltransferase activity and for investigating the integration between the biosynthesis of phosphatidylcholine and that of phosphatidylglycerol.

We thank Ms. N. E. Tyler, Mr. J. A. Head and Mr. G. R. Rader for expert technical assistance and Ms. D. Tutton for assistance in preparing this manuscript. This investigation was supported by grants (HD13912 and HD14373) from the National Institutes of Health. M. M. A. and G. C. Di R. were recipients of post-doctoral fellowships from the Robert A. Welch Foundation, Houston, Texas. M. D. V. was the recipient of a pre-doctoral summer fellowship from the Chilton Foundation, Dallas, Texas.

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