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# Cytidine monophosphate-dependent synthesis of phosphatidylglycerol in permeabilized type II pneumonocytes

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Results of previous investigations support the proposition that, in type II pneumonocytes, CMP is involved in integration of the synthesis of phosphatidylcholine and phosphatidylglycerol for lung surfactant. In the present investigation, the amount of CMP in rat type II pneumonocytes was altered directly and resultant changes in the synthesis of phosphatidylglycerol were examined. Type II pneumonocytes were made permeable to CMP by treatment with $Ca^{2+}$-free medium, and phosphatidylglycerol synthesis was then assessed by measurement of the incorporation of a radiolabelled precursor, [$^{14}$C]glycerol 3-phosphate, that was not effectively utilized by cells that resisted permeabilization. Incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol (but not into other lipids) was stimulated greatly by CMP (half-maximal stimulation at approx. 0.1 mM). CMP stimulated the incorporation of [$^{14}$C]glycerol 3-phosphate into both the phosphatidyl moiety and the head group of phosphatidylglycerol. Incorporation of [$^{14}$C]palmitate into phosphatidylglycerol was also stimulated by CMP. *myo*-Inositol, at concentrations found in foetal-rat serum (0.2–2.0 mM), inhibited CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol and promoted, instead, CMP-dependent incorporation into phosphatidylinositol. These data, when extrapolated to foetal type II pneumonocytes, are consistent with the view that the developmental increase in the synthesis of phosphatidylglycerol for surfactant by foetal lungs is promoted by the increase in intracellular CMP and the declining availability of *myo*-inositol that were found previously to be associated with this period of development.

## INTRODUCTION

Derivatives of cytidine nucleotides are intimately involved in the biosynthesis of phospholipids and gangliosides in mammalian cells. A co-product of these biosynthetic reactions is cytidine monophosphate (CMP). In several tissues, intracellular amounts of CMP are greatest when the rate of lipid synthesis is greatest, and CMP may be the most abundant cytidine nucleotide in tissues that are actively synthesizing lipids (Mandel & Edel-Harth, 1966; Quirk *et al.*, 1980; Anceschi *et al.*, 1984; Post *et al.*, 1984). Several investigators have proposed that CMP may have a direct or indirect regulatory function in lipid metabolism. Glycerol 3-phosphate acyltransferase activity in liver and brain is influenced by cytidine nucleotides (Fallon & Lamb, 1968; Zborowski & Wojtczak, 1969; Possmayer *et al.*, 1973). Possmayer (1974) examined the effects of various cytidine nucleotides on the acylation of glycerol 3-phosphate in rat brain and proposed that acylation may be regulated by cytidine nucleotides and would be greatest when the cytidylate energy charge was lowest. Similarly, Åkesson & Sundler (1977) concluded that CMP-dependent reversal of the biosynthetic reaction catalysed by cholinephosphotransferase in rat liver may be physiologically important.

During lung development in the foetal rabbit, an increase in the amount of intracellular CMP accompanies the increased production of phosphatidylcholine for lung surfactant (Quirk *et al.*, 1980). It was proposed (Quirk *et al.*, 1980; Bleasdale & Johnston, 1982*a*) that this increase in CMP in foetal lung tissue is responsible, in part, for the developmental decline in the amount of phosphatidylinositol in surfactant and the concomitant increase in the phosphatidylglycerol content of surfactant (Hallman & Gluck, 1980). CMP, at concentrations found in foetal rabbit lung tissue (Quirk *et al.*, 1980) or rat type II pneumonocytes (Anceschi *et al.*, 1984; Post *et al.*, 1984), promotes the incorporation of glycerol 3-phosphate into phosphatidylglycerol by lung microsomes (Bleasdale & Johnston, 1982*b*). In intact cells, CMP-dependent synthesis of phosphatidylglycerol appears to involve a stimulation of the reverse reaction catalysed by phosphatidylinositol synthase (EC 2.7.8.11) that results in increased availability of CDP-diacylglycerol (synthesized from phosphatidic acid) for phosphatidylglycerol biosynthesis (Bleasdale *et al.*, 1979; Bleasdale, 1985). When phosphatidylcholine synthesis by isolated type II pneumonocytes was stimulated, the intracellular concentration of CMP was approximately doubled and there was a selective increase in the synthesis of phosphatidylglycerol (Anceschi *et al.*, 1984). The objective of the present investigation was to alter directly the intracellular amount of CMP in isolated type II pneumonocytes and to examine any resultant changes in the synthesis of glycerolipids.

## MATERIALS AND METHODS

### Materials

The animals used in this investigation were male Sprague–Dawley rats (200–225 g) free from specific pathogens and were obtained from Sasco, Omaha, NE, U.S.A. Rats were housed inside a laminar-flow hood and were fed *ad libitum* until they were killed. Materials used in the procedure for isolating type II pneumonocytes were

obtained from the sources described by Mason *et al.* (1977*a*,*b*). L-[U-$^{14}$C]Glycerol 3-phosphate, disodium salt (144 Ci/mol) and [1-$^{14}$C]palmitic acid (59 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A. and ICN Radiochemicals, Irvine, CA, U.S.A., respectively. Cytidine 5′-monophosphate (sodium salt), 1-β-D-arabinofuranosylcytosine 5′-monophosphate (free acid), adenosine 5′-monophosphate (sodium salt), guanosine 5′-monophosphate (sodium salt), phospholipase D (EC 3.1.4.4) from peanut (*Arachis hypogoea*) (1 mg of protein catalysed the hydrolysis of 320 μmol of phosphatidylcholine/h at pH 5.6 and 30 °C), and coenzyme A (lithium salt) were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Lipids for use as chromatographic standards were obtained from Serdary Research Laboratories, London, Ontario, Canada. Pre-coated t.l.c. plates were from Analtech, Newark, DE, U.S.A. The sources of all other materials that were employed have been described previously (Bleasdale *et al.*, 1982; Anceschi *et al.*, 1984).

## Methods

**Isolation of rat type II pneumonocytes.** Type II pneumonocytes were isolated from the lungs of adult rats by use of a modification of the procedure of Mason *et al.* (1977*a*,*b*) as described elsewhere (Bleasdale *et al.*, 1983, 1985). The purity of the final cell suspension was assessed by optical microscopy with a modified Papanicolaou stain (Kikkawa & Yoneda, 1974). Approx. $5 \times 10^7$ cells, of which 75–85% were type II pneumonocytes, were isolated routinely from the lungs of six rats.

**Permeabilization of type II pneumonocytes.** Cells were permeabilized by use of a procedure based on that described by Streb & Schulz (1983) for the permeabilization of pancreatic acinar cells. Cells that were harvested after differential adherence (Bleasdale *et al.*, 1983) [approx. $5 \times 10^7$ cells in 50 ml of Dulbecco's modified Eagle's medium that contained 10% (v/v) foetal bovine serum] were collected by centrifugation (250 *g* for 6 min at 4 °C). The pellet of cells were suspended in 1.0 ml of ice-cold permeabilization medium [Hepes (10 mM, pH 7.4), containing KCl (135 mM), $MgCl_2$ (1 mM), $KH_2PO_4$ (1.2 mM) and EDTA (1 mM)]. The cells were collected immediately by centrifugation (250 *g* for 6 min at 4 °C) and were then resuspended in 1.0 ml of ice-cold permeabilization medium. Cells were again collected immediately (250 *g* for 6 min at 4 °C) and then were suspended in incubation medium [Hepes buffer (10 mM, pH 7.4), containing KCl (135 mM), $MgCl_2$ (1 mM), $KH_2PO_4$ (1.2 mM) and ATP (5 mM)]. This suspension of permeabilized cells (approx. $3 \times 10^7$ cells/ml) was used immediately after preparation as described below. Whenever permeabilized cells were incubated in the presence of radiolabelled precursors, other permeabilized cells were exposed concurrently to Trypan Blue dye. Permeabilization was assessed as the percentage of type II pneumonocytes that failed to exclude Trypan Blue dye.

**Incubation of permeabilized cells.** In most experiments, permeabilized cells (0.1 ml of a suspension that was prepared as described above) were incubated in medium [Hepes buffer (10 mM, pH 7.4), containing KCl (135 mM), $MgCl_2$ (1 mM), $KH_2PO_4$ (1.2 mM) and ATP (5 mM)] that was supplemented with [$^{14}$C]glycerol 3-phosphate (0.27 mM, 144 Ci/mol) and CMP (or other nucleotide) at various concentrations, in a total volume of 0.12 ml. In some experiments, [$^{14}$C]glycerol 3-phosphate was replaced by [$^{14}$C]palmitic acid (0.33 mM, 59 Ci/mol), CoA (0.1 mM) and bovine serum albumin (10 mg/ml). Cells were incubated in glass screw-cap culture tubes (150 mm × 20 mm) for 30 min at 37 °C.

**Extraction and separation of lipids.** Incubation of cells was terminated by the addition of 4.5 ml of chloroform/methanol (1:2, v/v) and 0.1 ml of HCl (6 M) to each incubation tube. Lipids were extracted from cells as described previously (Bleasdale *et al.*, 1979). Known amounts of various non-radiolabelled glycerophospholipids were added at the beginning of the extraction procedure to improve recovery of radiolabelled products and to facilitate their identification on thin-layer chromatograms. When [$^{14}$C]glycerol 3-phosphate was employed as the radiolabelled precursor, total lipid extracts were washed (Bleasdale *et al.*, 1979) and then subjected to two-dimensional t.l.c. as described by Yavin & Zutra (1977). When [$^{14}$C]palmitic acid was employed as the radiolabelled precursor, washed total lipid extracts were treated to remove non-esterified [$^{14}$C]palmitic acid before separation of lipid classes by use of two-dimensional t.l.c. Each total lipid extract was applied to a column (5 mm × 50 mm) of silica gel (Unisil; Clarkson Chemical Co., Williamsport, PA, U.S.A.) packed in chloroform. Non-esterified [$^{14}$C]palmitic acid was eluted from the column with chloroform (4.5 ml). Other lipids were eluted with chloroform/methanol (2:1, v/v) (2 ml) followed by chloroform/methanol (1:1, v/v) (3 ml). Lipids that were eluted by use of the chloroform/methanol mixtures were combined, evaporated to dryness under $N_2$, and subjected to two-dimensional t.l.c. (Yavin & Zutra, 1977). Neutral lipids in the total lipid extracts were separated by use of t.l.c. on silica-gel H plates that were developed in heptane/diethyl ether/acetic acid (75:25:4, by vol). Incorporation of radiolabelled precursors into individual lipid classes was measured by use of liquid-scintillation spectrometry as described previously (Bleasdale *et al.*, 1983).

**Analysis of the intramolecular distribution of $^{14}$C in phosphatidylglycerol.** Phosphatidyglycerol was separated from total lipid extracts as described above and located on the chromatogram by use of $I_2$ vapour. After the $I_2$ had evaporated, the area of the chromatogram that contained phosphatidylglycerol was removed and mixed with 2 ml of chloroform/methanol (1:1, v/v). The silica gel was collected by centrifugation (500 *g* for 10 min), the supernatant fluid was retained, and the silica-gel pellet was extracted twice more [2 × 2 ml of chloroform/methanol (1:1, v/v)]. The supernatant fluids (three) were combined, a portion was taken for measurement of radioactivity, and the remainder was evaporated to dryness under $N_2$. The residue was resuspended in 0.2 ml of Triton X-100 (1%, w/v) by use of ultrasound (Bransonic 220 bath–sonicator; 30 s at 125 W). The suspension was mixed with 0.2 ml of Tris/HCl buffer (100 mM, pH 8.0) that contained $CaCl_2$ (100 mM) and was incubated for 5 min at 37 °C. Phospholipase D (0.4 mg in 0.3 ml of 100 mM-Tris/HCl, pH 8.0) was then added and incubation was continued for a further 20 min at 37 °C. Incubations were terminated by the addition of 0.3 ml of EDTA (24 mM). Samples were then mixed well with 4.5 ml of chloroform/methanol (1:2, v/v) and

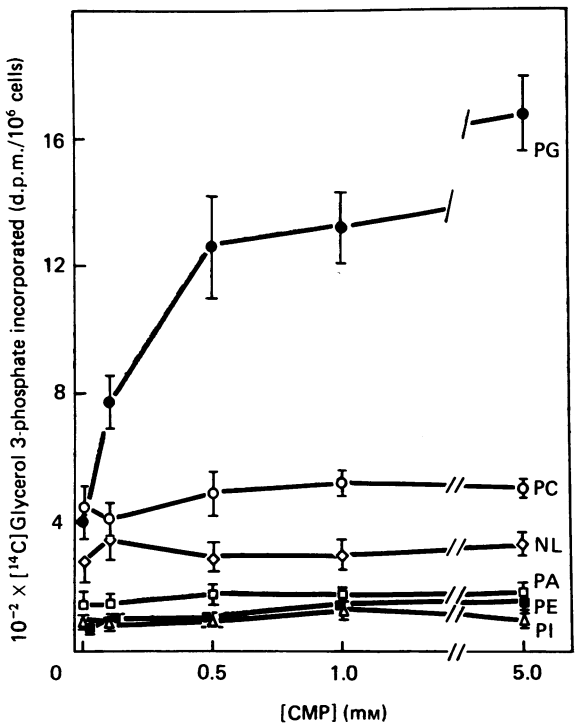


**Fig. 1. Effect of CMP on the incorporation of [$^{14}$C]glycerol 3-phosphate into various glycerolipids by permeabilized type II pneumonocytes**

Permeabilized type II pneumonocytes were suspended in 0.1 ml of $K^+$-rich medium that contained ATP (5 mM) (approx. $3 \times 10^6$ cells/tube). The cells were then incubated immediately at 37 °C for 30 min together with [$^{14}$C]glycerol 3-phosphate (0.27 mM, 144 Ci/mol) and CMP (at various concentrations). Incubations were terminated with the addition of 4.5 ml of chloroform/methanol (1:2, v/v) and lipids were extracted and separated as described in the Materials and methods section. The data are expressed as $^{14}$C (d.p.m.) incorporated into various glycerolipids by $10^6$ cells and are mean values ± S.E.M. that were derived from four experiments. Abbreviations: PG, phosphatidylglycerol; PC, phosphatidylcholine; NL, neutral lipids; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

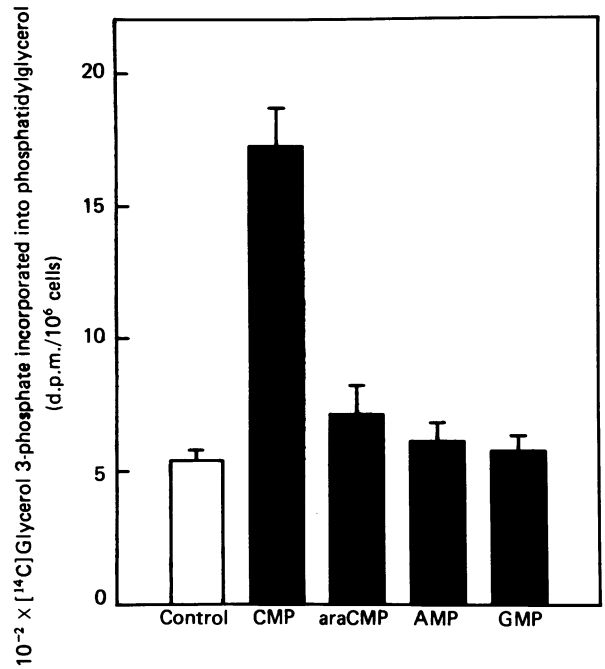


**Fig. 2. Effects of various nucleoside monophosphates on the incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by permeabilized type II pneumonocytes**

Permeabilized type II pneumonocytes (approx. $3 \times 10^6$ cells/tube) were incubated at 37 °C for 30 min in the presence of [$^{14}$C]glycerol 3-phosphate (0.27 mM, 144 Ci/mol) with or without various nucleoside monophosphates (1 mM) in a total volume of 0.12 ml as described in the Materials and methods section. Incubations were terminated, lipids were extracted, and phosphatidylglycerol was separated by use of two-dimensional t.l.c. The data are expressed as $^{14}$C (d.p.m.) incorporated into phosphatidylglycerol by $10^6$ cells and are mean values ± S.E.M. that they were derived from four experiments. Abbreviation: araCMP, 1-β-D-arabinofuranosylcytosine 5'-monophosphate.

0.1 ml of HCl (6 M). Chloroform (1.5 ml) and 1.5 ml of glycerol (0.1% in water) were added to each sample, which was then mixed and centrifuged (500 *g* for 10 min). After centrifugation, the upper phase (which contained [$^{14}$C]glycerol) was transferred to a scintillation vial, evaporated to dryness under $N_2$, and its radioactivity measured by use of liquid-scintillation spectrometry (Bleasdale *et al.*, 1979). The lower phase (which contained $^{14}$C-labelled lipids) was evaporated to dryness under $N_2$ and resuspended in 0.1 ml of chloroform/methanol (2:1, v/v). A portion (0.01 ml) of the lipid suspension was taken for measurement of radioactivity and the remainder was subjected to t.l.c. on silica-gel HL plates that were developed in chloroform/methanol/7 M-$NH_3$ (12:7:1, by vol.). Lipid spots on chromatograms were located with the use of $I_2$ vapour. After the $I_2$ had evaporated from chromatograms, radioactivity associated with various areas of the chromatogram was measured as described elsewhere (Bleasdale *et al.*, 1979). More than 90% of total radioactivity in the lower phase that was obtained after treatment with phospholipase D co-migrated with phosphatidic acid on thin-layer chromatograms.

**Other methods.** Statistical significance of differences between mean values was assessed by Student's *t* test for paired observations (Zar, 1974). Differences were considered significant at $P < 0.05$.

## RESULTS

In preliminary experiments, it was found that freshly isolated type II pneumonocytes were impermeable to CMP and that the incorporation of either [$^{14}$C]glycerol or [$^3$H]choline into lipids was unresponsive to extracellular CMP. For these reasons, freshly isolated type II pneumonocytes were made permeable to CMP by treatment with $Ca^{2+}$-free medium as described in the Materials and methods section. Cell permeability, assessed as the percentage of cells that took up Trypan Blue, increased from 9.6 ± 3.2% to 59.4 ± 2.5% after treatment with $Ca^{2+}$-free medium (mean values ± S.D., five experiments). Since approx. 40% of cells were not

**Table 1. Intramolecular distribution of $^{14}C$ in phosphatidylglycerol synthesized by permeabilized type II pneumonocytes that were incubated in either the absence or presence of CMP**

Type II pneumonocytes ($6 \times 10^6$) were permeabilized and then incubated for 30 min at 37 °C in the presence of [$^{14}C$]glycerol 3-phosphate (0.27 mM, 144 Ci/mol) with or without CMP (5 mM). Lipids were extracted and phosphatidylglycerol was separated from extracts by use of two-dimensional t.l.c. Phosphatidylglycerol was eluted from the chromatogram and treated with phospholipase D as described in the Materials and methods section. The products of the hydrolysis of phosphatidylglycerol were separated and $^{14}C$ associated with phosphatidic acid (phosphatidyl moiety) and with total water-soluble products (glycerol moiety) was measured. The data are from a typical experiment.

| | $^{14}C$ incorporated | | | |
|---|---|---|---|---|
| CMP (mM)... | 0 | | 5 | |
| | (d.p.m.) | (% of that recovered) | (d.p.m.) | (% of that recovered) |
| Phosphatidylglycerol | 3092 | 100 | 13662 | 100 |
| Phosphatidyl moiety | 769 | 24.1 | 2057 | 15.0 |
| Glycerol moiety | 2424 | 75.9 | 11698 | 85.0 |
| Total recovery of $^{14}C$ (%)... | | 103 | | 101 |

**Table 2. Effect of CMP on incorporation of [$^{14}C$]palmitate into glycerophospholipids by permeabilized type II pneumonocytes**

Type II pneumonocytes were permeabilized in $Ca^{2+}$-free medium and then incubated for 30 min at 37 °C in the presence of [$^{14}C$]palmitate (0.33 mM, 59 Ci/mol) and CMP at various concentrations. Lipids were extracted from cells and separated by use of two-dimensional t.l.c. Incorporation of $^{14}C$ into glycerophospholipids is expressed as d.p.m./$10^6$ cells and the data are mean values ± S.E.M. that were derived from four experiments. Statistical significance of effects of CMP on incorporation of [$^{14}C$]palmitate into various glycerophospholipids (different from incorporation of [$^{14}C$]palmitate in the absence of added CMP): * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$; † $P > 0.05$. When *non-permeabilized* type II pneumonocytes were suspended in Dulbecco's modified Eagle's medium, after 30 min at 37 °C, the incorporation of [$^{14}C$]palmitate (0.33 mM, 59 Ci/mol) into phosphatidylglycerol in the presence of CMP (1 mM) (8677 ± 1936 d.p.m./$10^6$ cells) or CMP (5 mM) (8321 ± 554 d.p.m./$10^6$ cells) was not significantly different from that observed in the absence of added CMP (6788 ± 250 d.p.m./$10^6$ cells) (mean values ± S.E.M. derived from three determinations).

| | | $^{14}C$ incorporated (d.p.m./$10^6$ cells) | | |
|---|---|---|---|---|
| Lipid | Concn. of added CMP (mM)... | 0 | 1 | 5 |
| Phosphatidylcholine | | 28264 ± 2725 | 31890 ± 4058† | 40731 ± 7684† |
| Phosphatidylglycerol | | 3228 ± 617 | 6578 ± 1666* | 8107 ± 766*** |
| Phosphatidylethanolamine | | 836 ± 131 | 919 ± 152† | 1454 ± 62** |
| Phosphatidylinositol | | 352 ± 70 | 455 ± 168† | 831 ± 217† |

permeabilized by this treatment, quantification of lipid synthesis was conducted routinely by using a radiolabelled precursor ([$^{14}C$]glycerol 3-phosphate) that was utilized effectively by permeabilized cells but not by non-permeabilized cells. Incorporation of [$^{14}C$]glycerol 3-phosphate into total lipids by non-permeabilized cells after 30 min of incubation at 37 °C was only 9% of that observed for permeabilized cells.

Treated cells incorporated [$^{14}C$]glycerol 3-phosphate into lipids at a constant rate for 30 min, at which time phosphatidylcholine and phosphatidylglycerol were the most radiolabelled lipids (Fig. 1). Incorporation of [$^{14}C$]glycerol 3-phosphate into phosphatidylglycerol increased greatly as the concentration of CMP in the incubation medium was increased from 0 to 0.5 mM; further small increases were observed as the concentration of CMP was increased from 0.5 to 5.0 mM. This effect of CMP was half-maximal at a CMP concentration of approx. 0.1 mM. Incorporation of [$^{14}C$]glycerol 3-phosphate into other glycerolipids was not altered significantly by CMP (1 mM) (Fig. 1). Other nucleoside monophosphates, including 1-β-D-arabinofuranosylcytosine 5′-monophosphate, were unable to substitute for CMP in stimulating the incorporation of [$^{14}C$]glycerol 3-phosphate into phosphatidylglycerol (Fig. 2).

The intramolecular distribution of $^{14}C$ incorporated into phosphatidylglycerol was next investigated to determine whether CMP-dependent incorporation of radioisotope reflected synthesis or head-group exchange. Phosphatidylglycerol was separated from total lipids by use of two-dimensional t.l.c. and then eluted from the silica gel. The phosphatidylglycerol was treated with phospholipase D and the radioactivity recovered in water-soluble products and in phosphatidic acid was measured as described in the Materials and methods section. As expected, after short periods of incubation (30 min) in the presence of [$^{14}C$]glycerol 3-phosphate, the radiolabel that was incorporated into phosphatidyl-

**Table 3. Effect of *myo*-inositol on CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by permeabilized type II pneumonocytes**

Type II pneumonocytes (approx. $6 \times 10^6$ cells per experimental condition) were permeabilized and then incubated for 30 min at 37 °C in the presence of [$^{14}$C]glycerol 3-phosphate (0.27 mM, 144 Ci/mol) with or without CMP (1 mM) and *myo*-inositol (0.2 mM or 2.0 mM). Lipids were extracted and then separated by use of two-dimensional t.l.c. Data are expressed as $^{14}$C (d.p.m.) incorporated into phosphatidylglycerol, phosphatidylinositol or phosphatidylcholine per $10^6$ cells and are mean values ± S.E.M. derived from four determinations. Statistical significance of comparisons: different from control (no addition), * $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$; different from addition of CMP (1 mM), † $P < 0.05$, †† $P < 0.025$, ††† $P < 0.01$.

| Addition | $^{14}$C incorporated (d.p.m./$10^6$ cells) into: | | |
|---|---|---|---|
| | Phosphatidylglycerol | Phosphatidylinositol | Phosphatidylcholine |
| None | 826 ± 38 | 171 ± 11 | 582 ± 50 |
| CMP (1 mM) | 1729 ± 225** | 225 ± 23 | 695 ± 49 |
| *myo*-Inositol | | | |
| (i) 0.2 mM | 675 ± 87 | 203 ± 26 | 557 ± 66 |
| (ii) 2.0 mM | 405 ± 26*** | 312 ± 30* | 570 ± 41 |
| CMP (1 mM) plus *myo*-inositol | | | |
| (i) 0.2 mM | 1512 ± 162** | 275 ± 23**,† | 692 ± 47 |
| (ii) 2.0 mM | 760 ± 178††† | 447 ± 39***,†† | 611 ± 54 |

glycerol by permeabilized cells was recovered largely in the head group of the molecule (Table 1). Radioactivity was also recovered, however, in the phosphatidyl moiety of phosphatidylglycerol. CMP (5 mM) stimulated the incorporation of [$^{14}$C]glycerol 3-phosphate into both the head-group and the phosphatidyl moiety of phosphatidylglycerol. The effect of CMP on incorporation into the head group was approx. 80% greater than CMP-dependent incorporation into the phosphatidyl moiety.

Further support for the proposition that CMP did not merely stimulate exchange of the head group of phosphatidylglycerol was the finding that incorporation of [$^{14}$C]palmitate into phosphatidylglycerol also was increased by CMP (Table 2). The magnitude of the CMP-dependent increase in incorporation of [$^{14}$C]palmitate into phosphatidylglycerol was similar to that of the CMP-dependent increase in incorporation of [$^{14}$C]glycerol 3-phosphate into the phosphatidyl moiety of phosphatidylglycerol. Such a comparison is only approximate, however, because some of the [$^{14}$C]palmitate that is incorporated into phosphatidylglycerol by the treated cells is attributable to non-permeabilized cells in the treated population and, as mentioned above, these cells are unresponsive to CMP added to the incubation medium (Table 2). Incorporation of [$^{14}$C]palmitate into other glycerophospholipids was not increased significantly by CMP (1 mM). At a higher concentration (5 mM), however, CMP stimulated the incorporation of [$^{14}$C]palmitate into phosphatidylethanolamine. The magnitude of CMP-dependent incorporation of [$^{14}$C]palmitate into phosphatidylethanolamine was less than that into phosphatidylglycerol (Table 2).

The increase in the amount of phosphatidylglycerol and the concomitant decrease in the amount of phosphatidylinositol in surfactant that occurs during lung development is promoted by an increase in the amount of intracellular CMP and by a decrease in the availability of *myo*-inositol (Bleasdale, 1985). Therefore the influence of *myo*-inositol on CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by permeabilized type II pneumonocytes was investigated. *myo*-Inositol was employed at concentrations in the range of those measured in foetal-rat serum (Burton & Wells, 1974; Quirk *et al.*, 1984). In the absence of added CMP, the addition of *myo*-inositol (2.0 mM) to permeabilized cells resulted in decreased incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol and increased incorporation into phosphatidylinositol (Table 3). Furthermore, CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol was inhibited by *myo*-inositol (2.0 mM), which promoted, instead, CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylinositol. In the presence of *myo*-inositol (2.0 mM), the amount of CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylinositol that is gained is similar to the lost amount of CMP-dependent incorporation into phosphatidylglycerol after correction is made on the basis that phosphatidylglycerol contains two glycerol moieties and only approx. 20% of total $^{14}$C is recovered in the phosphatidyl group (Table 1). Neither *myo*-inositol nor CMP, singly or in combination, affected significantly the incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylcholine (Table 3).

## DISCUSSION

The results of previous investigations support the view that CMP is involved in integration of the synthesis of phosphatidylcholine and phosphatidylglycerol for surfactant (Bleasdale *et al.*, 1979; Quirk *et al.*, 1980; Bleasdale & Johnston, 1982*b*; Bleasdale, 1985). It was found previously that, when the intracellular amount of CMP in type II pneumonocytes was increased indirectly by stimulation of phosphatidylcholine synthesis, there was a selective increase in the synthesis of phosphatidylglycerol (Anceschi *et al.*, 1984). The objective of the present investigation was to alter directly the intracellular amount of CMP in isolated type II pneumonocytes and to examine any resultant changes in the synthesis of glycerolipids. CMP does not readily enter type II pneumonocytes, and lipid synthesis by freshly isolated cells was unaffected by extracellular CMP. Type II pneumono-

cytes became responsive to extracellular CMP, however, after treatment with $Ca^{2+}$-free medium in a procedure that was used previously to permeabilize pancreatic acinar cells (Streb & Schulz, 1983). In other experiments (results not shown), type II pneumonocytes were permeabilized by use of either saponin or lysophosphatidylcholine, but these agents greatly inhibited lipid synthesis in the treated cells. Although permeabilization in $Ca^{2+}$-free medium was apparently incomplete, it was possible to investigate selectively lipid metabolism in permeabilized cells by use of a radiolabelled precursor ([$^{14}$C]glycerol 3-phosphate) that was not readily utilized by non-permeabilized cells.

CMP increased specifically the incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by permeabilized type II pneumonocytes. The possibility that CMP-dependent incorporation was due to stimulation of exchange of the head group of either phosphatidylglycerol or phosphatidylglycerol phosphate was considered, since it was reported previously that phosphatidylglycerol phosphate synthase (EC 2.7.8.5) purified from *Escherichia coli* catalysed the CMP-dependent exchange of the glycerol 3-phosphate moiety of phosphatidylglycerol phosphate (Hirabayashi *et al.*, 1976). On the basis of the intramolecular distribution of incorporated $^{14}$C, however, it appeared that CMP stimulated both head-group exchange and synthesis of phosphatidylglycerol. This conclusion was supported by the finding that CMP also increased the incorporation of [$^{14}$C]palmitate into phosphatidylglycerol. The concentrations at which CMP influenced the incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by permeabilized type II pneumonocytes were similar to those found in untreated type II pneumonocytes that are actively synthesizing phosphatidylcholine (Anceschi *et al.*, 1984; Post *et al.*, 1984). The observation that CMP does not influence the incorporation of either [$^{14}$C]glycerol 3-phosphate or [$^{14}$C]palmitate into phosphatidylcholine discounts the proposition of Post *et al.* (1984) that, in type II pneumonocytes, CMP inhibits net synthesis of phosphatidylcholine by stimulation of the reverse reaction catalysed by cholinephosphotransferase. Similarly, the absence of CMP-dependent inhibition of phosphatidylglycerol synthesis supports the proposition that, *in vivo*, the reaction catalysed by phosphatidylglycerol phosphate synthase is far from equilibrium (presumably because of active hydrolysis of phosphatidylglycerol phosphate to phosphatidylglycerol) (Hirabayashi *et al.*, 1976).

Since other nucleoside monosphosphates could not substitute for CMP in stimulating the incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol, it seems unlikely that the response to CMP was attributable to less-specific effects of nucleotides, e.g. inhibition of CDP-diacylglycerol hydrolysis (Carter & Kennedy, 1966; Longmuir & Johnston, 1980). It was proposed previously that the mechanism of CMP-dependent synthesis of phosphatidylglycerol involves promotion of the reverse reaction catalysed by phosphatidylinositol synthase so that CDP-diacylglycerol (synthesized from phosphatidic acid) is utilized preferentially for phosphatidylglycerol synthesis. The failure of 1-$\beta$-D-arabinofuranosylcytosine 5′-monophosphate to stimulate phosphatidylglycerol synthesis is consistent with such a mechanism, since Raetz *et al.* (1977) observed that 1-$\beta$-D-arabinofuranosylcytosine 5′-diphosphate-*sn*-1,2-dipalmitoylglycerol supported the synthesis of phosphatidylinositol, but not of phosphatidylglycerol, by subcellular fractions of rat liver.

In the presence of CMP, total production of CDP-diacylglycerol was presumably increased, since increased incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol was not accompanied by decreased incorporation into phosphatidylinositol, and the incorporation into cardiolipin after 30 min remained negligible. It is not known whether stimulation of CDP-diacylglycerol production by CMP (in addition to the effect of CMP on CDP-diacylglycerol utilization) involves either a direct action on CTP:phosphatidic acid cytidylyltransferase (EC 2.7.7.41), or activation of glycerol 3-phosphate acyltransferase (EC 2.3.1.15) by reduction in the cytidylate energy charge, as proposed for rat brain by Possmayer (1974). Although CMP did not affect the recovery of [$^{14}$C]glycerol 3-phosphate in phosphatidic acid after 30 min (Fig. 1), the effect of CMP on the turnover of phosphatidic acid was not investigated.

On the basis of the results of this investigation, it is concluded that, when the amount of intracellular CMP in type II pneumonocytes is increased *directly*, there is increased synthesis of phosphatidylglycerol. These data complement the previous observation that, when intracellular CMP is increased *indirectly* by stimulation of phosphatidylcholine synthesis in isolated type II pneumonocytes, there is a selective increase in the synthesis of phosphatidylglycerol (Anceschi *et al.*, 1984). It remains to be determined if conclusions based on the present investigation of type II pneumonocytes from adult rats are applicable to foetal type II pneumonocytes. Nevertheless, the finding that *myo*-inositol inhibited the CMP-dependent incorporation of [$^{14}$C]glycerol 3-phosphate into phosphatidylglycerol by adult type II pneumonocytes is consistent with previous evidence that, in foetal type II pneumonocytes, the developmental increase in synthesis of phosphatidylglycerol is promoted not only by the elevation of intracellular CMP that is associated with increased synthesis of phosphatidylcholine (Quirk *et al.*, 1980), but also by the declining availability of *myo*-inositol to the developing lungs (Bleasdale *et al.*, 1982, 1983; Hallman *et al.*, 1982; Batenburg *et al.*, 1982; Bleasdale, 1985).

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