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myo-Inositol homeostasis in foetal rabbit lung

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In several species, lung maturation is accompanied by a decline in the phosphatidylinositol content of lung surfactant and a concomitant increase in its phosphatidylglycerol content. To examine the possibility that this developmental change is influenced by the availability of *myo*-inositol, potential sources of *myo*-inositol for the developing rabbit lung were investigated. On day 28 of gestation the *myo*-inositol content of foetal rabbit lung tissue ($2.3 \pm 0.5\,\mu$mol/g of tissue) was not significantly different from that of adult lung tissue but the activity of D-glucose 6-phosphate:1L-*myo*-inositol 1-phosphate cyclase (cyclase) in foetal lung tissue (81.0 ± 9.0 nmol·h^{-1}·g of tissue^{-1}) was higher than that found in adult lung tissue (23.2 ± 1.0 nmol·h^{-1}·g of tissue^{-1}). Day 28 foetal rabbit lung tissue was found also to take up *myo*-inositol by a specific, energy-dependent, Na^+-requiring mechanism. Half-maximal uptake of *myo*-inositol by foetal rabbit lung slices was observed when the concentration of *myo*-inositol in the incubation medium was $85\,\mu$M. When the *myo*-inositol concentration was 1 mM (but not $100\,\mu$M) the addition of glucose (5.5 mM) stimulated *myo*-inositol uptake. *myo*-Inositol uptake was observed also in adult rabbit lung and was found to be sub-maximal at the concentration of *myo*-inositol found in adult rabbit serum. The concentration of *myo*-inositol in the serum of pregnant adult rabbits ($47.5 \pm 5.5\,\mu$M) was significantly lower than that of non-pregnant adult female rabbits ($77.9 \pm 9.2\,\mu$M). On day 28 of gestation the concentration of *myo*-inositol in foetal serum ($175.1 \pm 12.0\,\mu$M) was much less than on day 25, but more than that found on day 30. A transient post-partum increase in the concentration of *myo*-inositol in serum was followed by a rapid decline. Much of the *myo*-inositol in foetal rabbit serum probably originates from the placenta, where on day 28 of gestation a high cyclase activity (527 ± 64 nmol·h^{-1}·g of tissue^{-1}) was measured. The gestational decline in serum *myo*-inositol concentration, together with the decreasing cyclase activity of the lungs, is consistent with the view that maturation of the lungs is accompanied by decreased availability of *myo*-inositol to this tissue.

During foetal lung development in several species, there is a change from the production of a lung surfactant rich in phosphatidylinositol to one rich in phosphatidylglycerol (Hallman *et al.*, 1975; Hallman & Gluck, 1975, 1980). Human infants delivered before the appearance of phosphatidylglycerol-rich surfactant are at an increased risk of succumbing to respiratory distress syndrome of the newborn (Hallman *et al.*, 1976; Cunningham *et al.*, 1978). The control of the phosphatidylglycerol content of lung surfactant is not understood, but the reciprocal changes in the amounts of phosphatidylinositol and phosphatidylglycerol in surfactant are suggestive of regulation at the level of a common precursor of these two lipids. CDP-diacylglycerol is the precursor of both phosphatidylinositol and phosphatidylglycerol and is found in only small amounts in most mammalian cells (White, 1973). There is evidence that limited availability of CDP-diacylglycerol may restrict the biosynthesis of both phosphatidylinositol and phosphatidylglycerol and that under some circumstances there exists competition between these two biosynthetic pathways for the limited amount of CDP-diacylglycerol available (Freinkel *et al.*, 1975; Eichberg *et al.*, 1979; Hallman & Epstein, 1980; Esko & Raetz, 1980). In several studies *in vitro* employing various tissues, it has been observed that when the extracellular

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concentration of *myo*-inositol was increased, phosphatidylinositol biosynthesis was enhanced at the expense of phosphatidylglycerol biosynthesis (Eichberg *et al.*, 1979; Esko & Raetz, 1980). We (Quirk *et al.*, 1981) and others (Hallman & Epstein, 1980) have proposed that *myo*-inositol availability in the developing lung may be an important factor in the regulation of lung surfactant composition. However, *myo*-inositol homeostasis in the foetus of any species is not well understood. Campling & Nixon (1954) observed that in several species the concentration of *myo*-inositol in foetal blood is much greater than that in maternal blood. It was observed that the concentration of *myo*-inositol in rabbit blood declined during late gestation. Following a transient post-partum increase in the concentration of *myo*-inositol in neonatal blood, blood levels of *myo*-inositol continued to decline during neonatal development. In that study the source of *myo*-inositol for the foetal rabbit was not identified. However, in the sheep neither placental synthesis nor placental transport of *myo*-inositol could be detected and it was concluded that the foetal sheep is capable of supplying its own *myo*-inositol (Campling & Nixon, 1954; Nixon, 1968). Burton & Wells (1974) observed that foetal rat plasma contained *myo*-inositol at a concentration almost 10 times that found in the plasma of the dam. At the time of birth the level of *myo*-inositol in foetal rat plasma fell dramatically and was followed by a more gradual decrease during early life. The high concentration of *myo*-inositol in the foetal rat plasma appeared to be a reflection of greater biosynthesis of *myo*-inositol in the foetus than in the adult rat. The specific activity of the rate-limiting enzyme in *myo*-inositol biosynthesis (*myo*-inositol 1-phosphate synthase, D-glucose 6-phosphate:1L-*myo*-inositol 1-phosphate cyclase, EC 5.5.1.4) (cyclase) was found to be 10-fold higher in foetal rat liver than in adult rat liver (Burton & Wells, 1974). Indeed, in the foetus, the liver accounted for almost half of the total body cyclase activity and it is probable that much of the *myo*-inositol in foetal rat plasma originates in the foetal liver. Foetal tissues that synthesize little or no *myo*-inositol may obtain *myo*-inositol from the foetal blood by an uptake mechanism. *myo*-Inositol uptake mechanisms in a number of adult tissues from various species have been described (Hauser, 1965; Caspary & Crane, 1970; Varma *et al.*, 1970; Spector & Lorenzo, 1975). However, the capacity of foetal lung tissue for either *myo*-inositol synthesis or *myo*-inositol uptake remains unknown. As part of a study of the importance of *myo*-inositol in the regulation of lung surfactant composition in the foetus, the present investigation was undertaken in order to identify potential sources of *myo*-inositol for the developing lungs of the foetal rabbit during late gestation.

Experimental

Materials

Time-bred New Zealand White rabbits were obtained from Rich-Glo Lab Animals, El Campo, TX, U.S.A. and were fed *ad libitum* until killed by the intravenous administration of Nembutal (120mg/kg). Adult male Sprague–Dawley rats were purchased from Charles River Breeding Labs, Wilmington, MA, U.S.A. and were fed *ad libitum* until killed by decapitation. D-[1-^{14}C]Mannitol (45mCi/mmol), *myo*-[2-^{3}H]inositol (12.5Ci/mmol), inulin-[^{14}C]carboxylic acid (2mCi/g) and D-[1-^{14}C]-glucose 6-phosphate, disodium salt (57mCi/mmol) were obtained from New England Nuclear. D-Glucose 6-phosphate (disodium salt), *myo*-inositol, trimethylsilyl-*myo*-inositol, D-mannitol, D-sorbitol, sodium pyruvate and dihydroxyacetone were products of Sigma Chemical Company. L-Lactic acid was obtained from Mallinckrodt. Pyridine and *N*-trimethylsilylimidazole were supplied by Regis Chemical Company, Morton Grove, IL, U.S.A. β-NAD (grade 1) was purchased from Boehringer. $(NH_4)_2SO_4$ (Ultra Pure) was from Schwarz Mann, Orangeburg, NY, U.S.A. and insulin (bovine, crystalline) was from Eli Lilly. Phosphatidylinositol (from porcine liver) was obtained from Serdary Research Laboratories, London, Ontario, Canada. All other chemicals were commercially available reagent grade.

Measurement of the myo-inositol content of tissues

myo-Inositol was measured by g.l.c. chromatography of its trimethylsilyl derivative. For the derivatization, tissue samples were processed as follows. Blood serum (0.25ml) was mixed with a trace amount of *myo*-[2-^{3}H]inositol (approx. 4pmol, 0.05μCi) and serum proteins were precipitated as described by Somogyi (1945). The resulting supernatant fraction was applied to a column (1cm × 4cm) of mixed bed ion-exchange resin (Bio-Rad AG501-X8). *myo*-Inositol was eluted from the column by using two bed-volumes of water. The eluted fraction containing *myo*-inositol was then lyophilized.

For extraction of all other tissues, portions were homogenized in 5vol. of ice-cold $HClO_4$ (3%, v/v) using a Potter–Elvehjem Teflon/glass homogenizer. Each homogenate was centrifuged (12000*g* for 15min) and the resulting supernatant fraction was retained. The pellet was re-extracted with another 5vol. of $HClO_4$ (3%, v/v) as above. The resulting pellet was dissolved in NaOH (1M) and retained for subsequent determination of protein. The two $HClO_4$ extracts were combined and 5ml of this was mixed with a trace amount of *myo*-[2-^{3}H]inositol (approx. 4pmol, 0.05μCi) and neutralized by mixing with an equal volume of tri-*N*-octylamine (0.5M)

in Freon. The pH of the extract was monitored and if found to be less than pH 5 the neutralization procedure was repeated. The neutralized extract was deionized by using a column of mixed-bed ion-exchange resin as described above. The eluted column fraction containing *myo*-inositol was lyophilized. *myo*-Inositol in the lyophilized extracts of serum and other tissues was silylated employing a modification of the procedure of Brittain *et al.* (1971). To each lyophilized extract (in 150mm × 20mm glass culture tubes with Teflon-lined screw caps) were added 0.2ml of pyridine and 0.2ml of trimethylsilylimidazole. After 16h at room temperature, 2.0ml of water was added to each reaction mixture and derivatized products were extracted with three 2ml portions of hexane. The hexane extracts (three) were combined, evaporated under N_2, the residue was redissolved in 0.1ml of hexane and portions were taken for g.l.c. analysis (in duplicate) as described by Sweeley *et al.* (1963) and for liquid-scintillation spectrometry (Bleasdale *et al.*, 1979). For the g.l.c. analysis, trimethylsilyl α-methyl mannoside was employed as the internal standard and the amount of trimethylsilyl-*myo*-inositol in each extract was computed by measurement of peak area relative to the peak areas obtained with known amounts of authentic trimethylsilyl-*myo*-inositol. The values of *myo*-inositol content/g of tissue were all corrected for total recovery of the tracer *myo*-[2-^{3}H]inositol added to the initial extract. Total recovery of *myo*-[2-^{3}H]inositol throughout the procedure was usually 67–75% of that added initially.

Assay of D-*glucose 6-phosphate:1*L-*myo-inositol 1-phosphate cyclase activity*

Activity of D-glucose 6-phosphate:1L-*myo*-inositol 1-phosphate cyclase was measured in 0–40% $(NH_4)_2SO_4$ precipitates prepared from the 105000***g*** supernatant fractions of various tissues (Burton & Wells, 1974) employing the procedure of Barnett *et al.* (1970) as modified by Burton & Wells (1974).

Measurement of myo-[2-^{3}*H*]*inositol uptake by lung tissue*

Pregnant New Zealand White rabbits were killed on day 28 of gestation and foetuses were removed individually from uteri and decapitated before the lungs filled with air. Foetal lungs were removed rapidly and were combined with those of littermates. The lungs were rinsed with ice-cold NaCl (0.154M), blotted dry with filter paper and weighed. Strips (approx. 0.2cm × 1cm) of foetal lung tissue were cut and mounted on filter paper (Whatman 541, double-thickness) which had been soaked with ice-cold NaCl (0.154M). The strips of lung tissue were cut into slices (0.5mm thick) using a McIlwain Tissue Chopper (H. Mickle, Gomshall, Surrey, U.K.). The lung slices prepared from one litter of foetuses (2–7g) were incubated for 20min at 37°C in 10ml of Krebs–Henseleit original Ringer bicarbonate solution (Krebs & Henseleit, 1932). In some experiments the Krebs–Henseleit medium was modified to include glucose (5.5mM). Following incubation, lung slices were collected by vacuum filtration and 100mg portions were placed in individual glass liquid-scintillation vials (50mm × 27mm) which had been made gas-tight by wrapping Parafilm over the screw-threads of each vial. Krebs–Henseleit medium (0.95ml) was added to each vial and the air in each vial was replaced with O_2/CO_2 (19:1). The vials containing lung slices were incubated at 37°C for 15min in a Dubnoff metabolic shaker. After 15min of incubation, 0.05ml of Krebs–Henseleit medium containing *myo*-[2-^{3}H]-inositol (2mM, approx. 0.05μCi/nmol) and [1-^{14}C]-mannitol (carrier-free, approx. 0.15mM, 0.045μCi/nmol) was added to each vial. Following incubation for a further 40min at 37°C the lung slices were collected on glass-fibre filters (Whatman SG/A) by vacuum filtration and were washed with two 1ml portions of ice-cold Krebs–Henseleit medium. The lung slices were then weighed and homogenized in 1ml of ice-cold $HClO_4$ (3%, v/v) using a Potter–Elvehjem Teflon/glass homogenizer. The $HClO_4$ extracts were centrifuged (7500***g*** for 10min in an Eppendorf model 5413 centrifuge) and aliquots (0.1ml) of each supernatant fraction were added to 0.75ml of water for the measurement of ^{3}H and ^{14}C by liquid-scintillation spectrometry using a scintillation mixture containing Triton X-100 (Bleasdale *et al.*, 1979), making corrections for quenching. In some experiments, portions (0.1ml) of the incubation medium (i.e. the filtrates resulting after collection of the lung slices) were also taken for liquid-scintillation spectrometry. [1-^{14}C]Mannitol was not taken up by the lung slices and was used to measure the amount of *myo*-[2-^{3}H]inositol associated with the extracellular space. In initial experiments we found that the extracellular space measured employing [1-^{14}C]mannitol was the same as that measured employing inulin-[^{14}C]carboxylic acid. The extracellular space measured by using [1-^{14}C]mannitol was independent of the concentration of mannitol in the medium. To determine the amount of *myo*-[2-^{3}H]inositol taken up into the intracellular space, the following calculation was performed.

Intracellullar *myo*-[2-^{3}H]inositol = tissue *myo*-[2-^{3}H]inositol − [tissue [1-^{14}C]mannitol × (^{3}H per ml of medium/^{14}C per ml of medium)]

Unless stated otherwise, uptake of *myo*-inositol is expressed as nmol of *myo*-inositol taken up·h^{-1}·g of tissue^{-1}.

The procedure for measurement of *myo*-[2-^{3}H]-inositol uptake by adult rabbit lung tissue was as

described above for foetal rabbit lung tissue except that, in order to avoid contamination by large amounts of non-alveolar tissue, only marginal strips of tissue were used from adult lungs. In some experiments, the incorporation of *myo*-[2-^{3}H]inositol into phosphatidylinositol by lung slices was measured. For this purpose the lung slices were homogenized in ice-cold water (1 ml) instead of $HClO_4$ (3%, v/v) and lipids were extracted as described by Hajra *et al.* (1968).

Characterization of myo-[2-^{3}H]inositol and phosphatidyl[2-^{3}H]inositol

Following incubation with *myo*-[2-^{3}H]inositol, lung slices were homogenized either in ice-cold $HClO_4$ (3%, v/v) or ice-cold water as described above. For the separation of *myo*-[2-^{3}H]inositol, portions of the neutralized $HClO_4$ extracts were subjected to ascending paper chromatography (Lewin *et al.*, 1974). Lipid analyses were performed on lung slices homogenized in water. Lipids were extracted and phosphatidyl[2-^{3}H]inositol was separated by one-dimensional and two-dimensional t.l.c. as described elsewhere (Bleasdale *et al.*, 1979).

Other methods

DNA was measured by using the procedure of Burton (1956). Protein measurements were performed according to Lowry *et al.* (1951) using bovine serum albumin as the reference standard. Where appropriate, data were subjected to an analysis of variance. Differences between mean values were determined by the Newman–Keuls multiple range comparison test (Zar, 1974). The Kruskal–Wallis H statistic was utilized to test for differences between groups when non-parametric analysis was necessary (Linton & Gallo, 1975).

Results

In the foetal rabbit (as in the human foetus) the ability to synthesize and secrete lung surfactant does not appear until late in gestation. Increased rates of phosphatidylcholine biosynthesis in foetal rabbit lung are observed first at about day 26 of gestation (term is day 31) and the developmental change from a surfactant rich in phosphatidylinositol to one rich in phosphatidylglycerol does not occur until the time of birth (Hallman & Gluck, 1980). For these reasons we investigated *myo*-inositol homeostasis in the foetal rabbit during late gestation.

myo-Inositol content of adult and foetal rabbit tissues

The amount of *myo*-inositol found in foetal rabbit lung tissue on day 28 of gestation ($2.3 \pm 0.5\,\mu$mol/g of tissue, mean ± S.E.M., $n = 11$) was not significantly greater than that found in adult rabbit lung tissue ($1.9 \pm 0.3\,\mu$mol/g of tissue, $n = 6$). Likewise, the amount of *myo*-inositol found in foetal rabbit liver tissue on day 28 of gestation ($0.8 \pm 0.15\,\mu$mol/g of tissue, $n = 8$) was not significantly greater than that found in adult rabbit liver tissue ($0.5 \pm 0.1\,\mu$mol/g of tissue, $n = 5$).

On day 25 of gestation the concentration of *myo*-inositol in foetal rabbit serum was found to be more than five times that in the maternal serum ($P < 0.01$) (Fig. 1). In agreement with Campling & Nixon (1954) we observed that the concentration of *myo*-inositol in foetal rabbit serum decreased during gestation (Fig. 1). However, in contrast with previous measurements of plasma *myo*-inositol concentration in neonatal rat (Burton & Wells, 1974) we observed a post-partum increase in the serum concentration of *myo*-inositol in the neonatal rabbit, a finding that confirms the earlier observation of Campling & Nixon (1954). The concentration of *myo*-inositol in the serum of pregnant rabbits on day 28 of gestation was

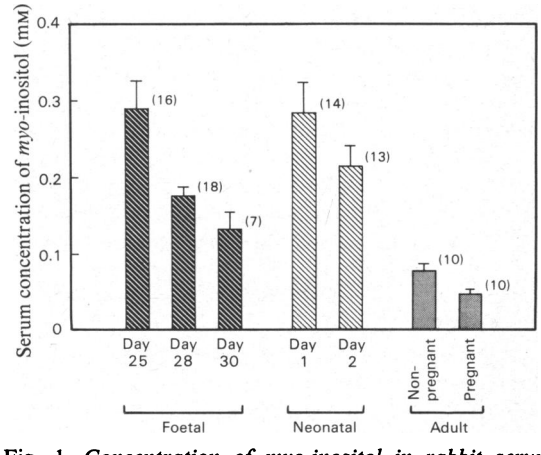

Fig. 1. *Concentration of myo-inositol in rabbit serum during development*

myo-Inositol in rabbit serum was measured by g.l.c. of its trimethylsilyl derivative as described in the Experimental section. The data shown are mean values ± S.E.M. for the number of animals shown in parentheses. Differences between mean values were determined by the Newman–Keuls multiple range comparison test. The concentration of *myo*-inositol in pregnant rabbit serum on day 28 of gestation was significantly lower ($P < 0.025$) than in serum from non-pregnant rabbits. The concentration of *myo*-inositol in adult (pregnant and non-pregnant) rabbit serum is lower than at any time in the foetal or neonatal period ($P < 0.001$). There was a significant ($P < 0.001$) decrease in the concentration of *myo*-inositol in foetal rabbit serum between day 25 and day 30 of gestation. Serum levels of *myo*-inositol in neonatal rabbits (day 1 or day 2) was not statistically different from the levels found in the serum of foetal rabbits on day 25 of gestation.

significantly lower ($P < 0.025$) than that in non-pregnant rabbits ($47.5 \pm 5.5\,\mu$M versus $77.9 \pm 9.2\,\mu$M, respectively).

D-Glucose 6-phosphate:1L-myo-inositol 1-phosphate cyclase activity in rabbit tissues

Possible sources of the large amount of *myo*-inositol in foetal rabbit serum were investigated. There is evidence that in many tissues the rate-limiting reaction in the biosynthesis of *myo*-inositol from glucose is that catalysed by D-glucose 6-phosphate:1L-*myo*-inositol 1-phosphate cyclase (cyclase) (Eisenberg, 1967). We measured cyclase activity in 0–40% $(NH_4)_2SO_4$ precipitates of the 105000 *g* supernatant fractions prepared from lung, liver and placenta. On day 28 of gestation, foetal rabbit lung was found to synthesize *myo*-inositol 1-phosphate at a rate almost four times that of maternal lung (Table 1). Foetal rabbit liver synthesized *myo*-inositol 1-phosphate at a rate much slower than that of foetal lung, but greater than that of maternal liver. For comparison, we measured cyclase specific activity in a mammalian tissue in which activity of this enzyme has been reported to be high, i.e. rat testes (Eisenberg, 1967). We found that under the conditions employed in this investigation, the specific activity of cyclase in adult rat testes was $1.4\,\mu$mol·h^{-1}·g of tissue^{-1}, or approx. 17 times that measured in foetal rabbit lung. However, we observed also that the rabbit placenta, on day 28 of gestation, had an unexpectedly high specific activity of cyclase (Table 1). These observations are in contrast to those of Burton & Wells (1974) who investigated *myo*-inositol biosynthesis in the foetal rat. Foetal rat liver was found to have a large amount of cyclase activity which declined during development. Furthermore, the rat placenta was found to have only low activity of cyclase (Burton & Wells, 1974). We confirmed the observation of Burton & Wells (1974) that foetal rat liver does have a high specific activity of cyclase (results not shown).

Table 1. *Activity of D-glucose 6-phosphate:1L-myo-inositol 1-phosphate cyclase in rabbit placenta, foetal and adult rabbit lung and liver*

Employing the procedures described in the Experimental section, cyclase activity was measured in the 0–40% $(NH_4)_2SO_4$ precipitates prepared from 105000 *g* supernatant fractions of the tissues listed below. The data are mean values ± S.E.M. for the number of determinations shown in parentheses. Foetal lungs (livers) from littermates were combined for the assay of cyclase activity and the value given in parentheses is the number of litters analysed.

Tissue	Cyclase activity (nmol·h^{-1}·g of tissue^{-1})
Day 28 foetal lung	81.0 ± 9.0 (7)
Adult lung	23.2 ± 1.0 (3)
Day 28 foetal liver	17.5 ± 3.3 (6)
Adult liver	7.6 ± 2.1 (4)
Day 28 placenta	527.0 ± 64.0 (6)

Uptake of myo-inositol by foetal rabbit lung

In view of the level of activity of cyclase measured in foetal rabbit lung we investigated the possibility that this foetal tissue obtains a substantial amount of *myo*-inositol from the blood. For measurement of *myo*-inositol uptake we quantified the amount of *myo*-[2-^{3}H]inositol taken up by slices (0.5 mm thick) of foetal rabbit lung obtained on day 28 of gestation. To correct for *myo*-[2-^{3}H]inositol associated with the extracellular space, [1-^{14}C]mannitol was employed as described (Experimental section). We found that *myo*-[2-^{3}H]inositol entered rapidly into the extracellular space and after less than 2 min the amount of *myo*-[2-^{3}H]inositol in the extracellular space remained constant for the remainder of the standard incubation period (40 min). The increase in *myo*-[2-^{3}H]inositol in the extracellular space observed after 120 min (Fig. 2) may have resulted from the tissue swelling as a consequence of the detrimental effects of prolonged incubation. Uptake of radiolabel into the intracellular space was also observed (Fig. 2). After 120 min of incubation, when the water-soluble radiolabelled compounds in the intracellular space were analysed by paper chromatography, more than 95% of recovered ^{3}H was found

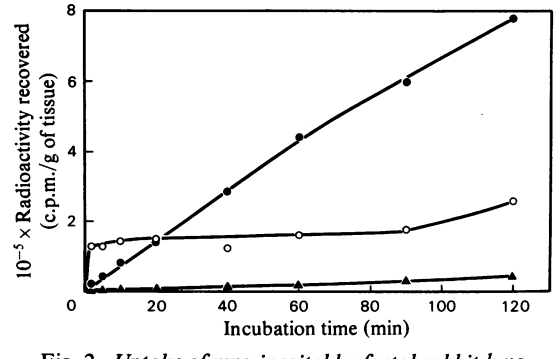

Fig. 2. *Uptake of myo-inositol by foetal rabbit lung*
Slices of day 28 foetal rabbit lung were prepared and then incubated in the presence of *myo*-[2-^{3}H]inositol and [^{14}C]mannitol as described in the Experimental section. ○, Uptake of *myo*-[2-^{3}H]inositol into the extracellular space; ●, uptake of *myo*-[2-^{3}H]inositol into the intracellular space; ▲, incorporation of *myo*-[2-^{3}H]inositol into phosphatidylinositol. Data are the average values derived from two experiments.

to co-migrate with authentic *myo*-inositol. During the course of a standard assay (40min) the rate of uptake of *myo*-[2-^{3}H]inositol into the intracellular space was constant. Even after 120min of incubation the rate of *myo*-[2-^{3}H]inositol uptake decreased only slightly (Fig. 2).

Incorporation of *myo*-[2-^{3}H]inositol into a total lipid extract by foetal rabbit lung slices was detected as early as 2min after exposure to the radiolabelled compound (Fig. 2). When the radiolabelled total lipid extract was subjected to either one-dimensional or two-dimensional t.l.c. only one radiolabelled component was detected and this co-chromatographed with authentic phosphatidylinositol. However, the extent of *myo*-[2-^{3}H]inositol incorporation into phosphatidylinositol remained small even after incubation for 120min. The most likely explanation for the low incorporation of *myo*-[2-^{3}H]-inositol into phosphatidylinositol by foetal rabbit lung slices is that the amount of *myo*-[2-^{3}H]-inositol taken up during the standard 40min incubation period (approx. 24nmol/g of tissue) is diluted by the relatively large amount of non-radiolabelled *myo*-inositol already present in the tissue (2.3μmol/g of tissue).

Uptake of *myo*-[2-^{3}H]inositol into foetal rabbit lung slices occurred by a saturable process (Fig. 3) and half-maximal uptake was observed at a *myo*-inositol concentration of 85μM. The uptake of *myo*-[2-^{3}H]inositol by this mechanism was not inhibited by the presence in the medium of mannitol, sorbitol or sucrose (Table 2). However, *myo*-inososose-2 (2L-2,4,6/3,5-pentahydroxycyclohexanone) and *myo*-inositol 2-phosphate were able to reduce the amount of *myo*-[2-^{3}H]inositol taken up into the intracellular space. When foetal lung slices were incubated with *myo*-[2-^{3}H]inositol (100μM) and *myo*-inositol 2-phosphate (1mM) for 40min, g.l.c. analysis of the medium after incubation revealed no increase in the concentration of free *myo*-inositol. Therefore, the effect of inositol 2-phosphate on *myo*-inositol uptake did not appear to be a result of extracellular hydrolysis of *myo*-inositol 2-phosphate with a consequent reduction in the specific radioactivity of *myo*-[2-^{3}H]inositol in the medium.

The uptake of *myo*-[2-^{3}H]inositol by foetal rabbit lung slices is inhibited by ouabain, 2,4-dinitrophenol plus 2-deoxyglucose and by cold (4°C) (Table 3), findings that are consistent with the proposition that this *myo*-inositol uptake system is energy-dependent, as it is in other tissues (Hauser, 1965; Caspary & Crane, 1970; Varma *et al.*, 1970; Spector & Lorenzo, 1975). Another common feature of *myo*-inositol uptake systems that have been described is that of a Na^+ requirement (e.g. Hauser, 1965). Likewise, the energy-dependent uptake of *myo*-[2-^{3}H]inositol by foetal rabbit lung slices was also found to be Na^+-requiring; K^+, Li^+ or $Tris^+$ were unable to substitute for Na^+ (Table 4). The effect of glucose on the uptake of *myo*-[2-^{3}H]inositol by foetal rabbit lung slices was investigated. Under standard

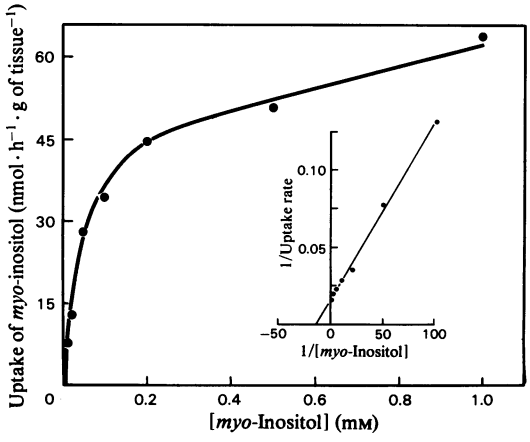

Fig. 3. *Dependence of myo-inositol uptake on the extracellular concentration of myo-inositol*

Uptake of *myo*-[2-^{3}H]inositol into the intracellular space of slices of day 28 foetal rabbit lung was measured as described in the Experimental section in the presence of *myo*-inositol at various concentrations. Data are the mean values derived from three experiments.

Table 2. *Effects of carbohydrates on myo-inositol uptake by slices of foetal rabbit lung*

Uptake of *myo*-[2-^{3}H]inositol into the intracellular space of day 28 foetal rabbit lung slices after 40min of incubation was measured as described in the Experimental section. Various carbohydrates, at the concentrations shown, were added to the incubation medium simultaneously with *myo*-[2-^{3}H]inositol. The incubation medium consisted of Krebs–Henseleit original Ringer bicarbonate solution supplemented with glucose (5.5mM) and contained *myo*-inositol at a concentration of 100μM. Data are the average values derived from two experiments.

Carbohydrate added (mM)	*myo*-Inositol uptake (nmol·h^{-1}·g of tissue^{-1})
None	30.0
myo-Inososose-2 (0.1)	24.0
myo-Inososose-2 (1.0)	13.8
D-Mannitol (0.1)	29.9
D-Mannitol (1.0)	33.2
D-Sorbitol (0.1)	30.0
D-Sorbitol (1.0)	27.0
Sucrose (1.0)	31.2
myo-Inositol 2-phosphate (0.1)	29.3
myo-Inositol 2-phosphate (1.0)	14.9

assay conditions, when the concentration of *myo*-[2-^{3}H]inositol was 100μM, uptake of *myo*-[2-^{3}H]-inositol was the same in incubation medium containing glucose (5.5 or 27.5mM) as it was in medium lacking glucose (results not shown). However, when the concentration of *myo*-[2-^{3}H]inositol in the incubation medium was 1mM a marked increase in *myo*-[2-^{3}H]inositol uptake was observed in the presence of glucose (5.5mM) (Table 5). The stimulation of *myo*-[2-^{3}H]inositol uptake by glucose was essentially unchanged when the glucose concentration of the incubation medium was increased from 5.5mM to 27.5mM. It may be that this effect of glucose on *myo*-[2-^{3}H]inositol uptake is not simply a result of correcting an energy deficiency in lung slices that have been incubated in medium lacking an energy source. While it appears that *myo*-[2-^{3}H]inositol uptake is an energy-dependent process (Table 3) it is also true that foetal rabbit lung slices in medium containing *myo*-[2-^{3}H]inositol (100μM), but lacking glucose, continue to take up *myo*-[2-^{3}H]inositol at a constant rate for at least 40min (Fig. 2). Furthermore, other potential sources of energy, when added to the incubation medium, were unable to substitute for glucose in stimulating *myo*-[2-^{3}H]inositol uptake by foetal lung slices (Table 5). We were unable to detect any reproducible effects of insulin (10^{-7}M) on glucose-stimulated uptake of *myo*-[2-^{3}H]inositol by foetal rabbit lung slices under the conditions described here (results not shown).

While the data concerning *myo*-inositol uptake were obtained with slices of foetal rabbit lung, many of the experiments were repeated employing adult rabbit lung tissue. When the rate of uptake of *myo*-[2-^{3}H]inositol by adult rabbit lung slices was compared with that of foetal rabbit lung slices employing a concentration of *myo*-[2-^{3}H]inositol (20μM) within the range found in adult rabbit serum, uptake by adult lung tissue was only 45% of that by foetal lung tissue. The faster rate of *myo*-[2-^{3}H]-inositol uptake by foetal lung tissue did not appear to be a result of a greater number of cells per g of tissue since, as stated previously, only marginal strips of adult lung tissue were employed in uptake experiments and the DNA content of these strips ($3.8 \pm 0.6\mu$g/g of tissue, mean $\pm$ S.E.M., $n = 5$) was not significantly different from the DNA content of day 28 foetal lung tissue ($4.1 \pm 0.5\mu$g/g of tissue, $n = 5$). The *myo*-inositol uptake system of adult rabbit lung exhibited the same energy-dependence, Na^+-requirement and specificity as did the *myo*-inositol uptake system of foetal rabbit lung. Furthermore, the affinities of the two systems for *myo*-inositol were similar (half-maximal uptake being observed at a concentration of *myo*-inositol of 85μM

Table 3. *Inhibitors of myo-inositol uptake by foetal rabbit lung slices*

Uptake of *myo*-[2-^{3}H]inositol into the intracellular space of day 28 foetal rabbit lung slices after 40min of incubation was measured as described in the Experimental section. The incubation medium was supplemented with glucose (5.5mM) and contained *myo*-inositol at a concentration of 100μM. Ouabain or 2,4-dinitrophenol plus 2-deoxyglucose were added at the beginning of the 15min preincubation period i.e. before the addition of *myo*-[2-^{3}H]inositol. To test the effect of cold on uptake of *myo*-inositol, foetal rabbit lung slices were pretreated at 37°C for 20min (see the Experimental section) and then preincubated (15min) at 4°C before being incubated (40min) in the presence of *myo*-[2-^{3}H]inositol at 4°C. Data are the average values derived from two experiments.

Addition to standard assay	*myo*-Inositol uptake (nmol·h^{-1}·g of tissue^{-1})
None	36.8
Ouabain (1mM)	5.9
2,4-Dinitrophenol (0.5mM) plus 2-deoxyglucose (0.5mM)	19.5
Cold (4°C)	0

Table 4. *Na^+-dependence of myo-inositol uptake by foetal rabbit lung slices*

Uptake of *myo*-[2-^{3}H]inositol by day 28 foetal rabbit lung slices was measured as described in the Experimental section. During the pretreatment (20min) of foetal rabbit lung slices unmodified Krebs–Henseleit medium was used. For the preincubation period (15min) and the incubation period (40min) the Krebs–Henseleit medium was supplemented with glucose (5.5mM) and was modified further as detailed below. The concentration of *myo*-inositol in each medium was 100μM. Data are the average values derived from two experiments.

Alteration in Krebs–Henseleit medium	*myo*-Inositol uptake (nmol·h^{-1}·g of tissue^{-1})
None	26.6
NaCl replaced by KCl	0
NaCl replaced by LiCl	9.0
NaCl replaced by Tris/HCl	7.2
NaCl and $NaHCO_3$ replaced by KCl and $KHCO_3$	0
NaCl and $NaHCO_3$ replaced by LiCl and $KHCO_3$	3.3
NaCl and $NaHCO_3$ replaced by Tris/HCl and $KHCO_3$	1.2

Table 5. *Effects of glucose, fructose, pyruvate, lactate and dihydroxyacetone on uptake of myo-inositol by foetal rabbit lung*
Uptake of *myo*-[2-^{3}H]inositol into the intracellular space of slices of day 28 foetal rabbit lung was measured as described in the Experimental section. Lung slices were pretreated (20 min) in Krebs–Henseleit original Ringer bicarbonate solution, then preincubated (15 min) in Krebs–Henseleit solution modified as detailed below, before the addition of *myo*-[2-^{3}H]inositol and [^{14}C]mannitol. Incubation with *myo*-[2-^{3}H]inositol was for 40 min and the concentration of *myo*-inositol in the medium was 1 mM. Data are the mean values ± S.E.M. derived from the number of experiments shown in parentheses. Differences between mean values were analysed using the Kruskal–Wallis H statistic. The addition of glucose (5.5 mM or 27.5 mM) increased significantly ($P < 0.01$) the uptake of *myo*-inositol by foetal rabbit lung slices. Other potential energy sources (pyruvate, lactate, dihydroxyacetone or fructose) could not substitute for glucose in stimulating uptake of *myo*-inositol.

Addition to Krebs–Henseleit medium	*myo*-Inositol uptake (nmol·h^{-1}·g of tissue^{-1})
None	35.0 ± 9.2 (6)
Glucose (5.5 mM)	62.5 ± 7.6 (6)
Glucose (27.5 mM)	64.7 ± 10.5 (5)
Pyruvate (5 mM)	32.4 ± 2.9 (5)
Lactate (5 mM)	25.6 ± 4.4 (4)
Pyruvate plus lactate (2.5 mM + 2.5 mM)	31.3 (2)
Dihydroxyacetone (2 mM)	23.6 ± 3.0 (3)
Fructose (5.5 mM)	28.3 ± 0.6 (3)

in the foetal lungs and 74 μM in the adult lungs). These findings are consistent with the view that the mechanism of *myo*-inositol uptake in foetal rabbit lung is the same as that in adult rabbit lung. However, *in vivo* the extent of *myo*-inositol uptake in foetal rabbit lung is probably much greater than that of adult rabbit lung. This obtains firstly because of the greater capacity (per g of tissue) for *myo*-inositol uptake by foetal lung compared with adult lung and secondly because early in gestation the serum concentration of *myo*-inositol in the foetus is sufficient to saturate the uptake system in the lung whereas in adult rabbit serum the concentration of *myo*-inositol is much less than that required for maximal uptake. Therefore, the decline in the serum concentration of *myo*-inositol that occurs during gestation (Fig. 1) may influence *myo*-inositol homeostasis in the foetal lung.

Discussion

As part of an investigation of the influence of *myo*-inositol on foetal lung surfactant composition the present study was undertaken to characterize potential sources of *myo*-inositol for the developing foetal rabbit lung. It was found that foetal rabbit lung on day 28 of gestation did not contain significantly more *myo*-inositol than did adult rabbit lung. Two potential sources of the *myo*-inositol in foetal rabbit lung were identified. First, foetal rabbit lung tissue had a limited capacity for *myo*-inositol biosynthesis and second, this tissue was able to take up *myo*-inositol from the extracellular medium by an energy-dependent, Na^+-requiring process.

In previous studies of *myo*-inositol biosynthesis in other mammalian tissues the rate-limiting reaction appears to be the conversion of glucose 6-phosphate to inositol 1-phosphate catalysed by D-glucose 6-phosphate:1L-*myo*-inositol 1-phosphate cyclase (cyclase) (Eisenberg, 1967). There is evidence that in foetal rat tissues, as in adult rat tissues, low cyclase activity limits biosynthesis of *myo*-inositol (Burton & Wells, 1974). Although glucose availability and hexokinase activity in foetal rabbit lung tissue were not determined, there is reason to believe that, as in other tissues, measurement of cyclase activity provides an index of *myo*-inositol biosynthesis. The activity of cyclase in foetal rabbit lung on day 28 of gestation was found to be small when compared with that in a tissue (rat testes) which actively synthesizes *myo*-inositol, but was much greater than that found in either foetal rabbit liver or adult rabbit lung. A developmental decrease in cyclase activity in both lung and liver was observed. The developmental change in cyclase activity may be regulated hormonally, since it was reported recently that in the adult male rat the activity of cyclase in primary and secondary sex organs is controlled by pituitary hormones and cyclase activity of the liver is regulated by thyroid hormone (Hasegawa & Eisenberg, 1981). The low activity of cyclase in foetal rabbit liver is in contrast to the high activity found in foetal rat liver (Burton & Wells, 1974). Almost half of the total cyclase activity of the foetal rat was associated with the liver and this foetal tissue probably is the source of much of the *myo*-inositol in the blood of the foetal rat (Burton & Wells, 1974). On the other hand, it appears that much of the *myo*-inositol in the blood of the foetal rabbit may originate in the placenta, where high activity of cyclase was measured. The high activity of cyclase in rabbit placenta was unexpected since rat placenta

contains little activity of this enzyme (Burton & Wells, 1974) and sheep placenta none (Campling & Nixon, 1954). Further investigation is required in order to identify the cell-type of the rabbit placenta that possesses high activity of cyclase and to determine the relative distribution of newly synthesized *myo*-inositol between maternal and foetal blood.

In addition to synthesizing *myo*-inositol, foetal rabbit lung is able to take up *myo*-inositol from the extracellular medium and the maximal rates for these two processes *in vitro* are similar. Uptake of *myo*-inositol by foetal rabbit lung shares several of the features of *myo*-inositol uptake in other tissues, including specificity, energy-dependence and Na^+ requirement. Furthermore, the characteristics of *myo*-inositol uptake by foetal rabbit lung were essentially the same as those of adult rabbit lung. However the capacity for *myo*-inositol uptake (per g of tissue) was greater in foetal lung than in adult lung. While this latter finding was not due to a larger number of cells per g of foetal lung, it may have been a result of a disparity in the types of cell found in day 28 foetal and adult lung (Wang *et al.*, 1971). The affinities for *myo*-inositol of the adult and foetal rabbit lung uptake systems were similar (half-maximal uptake at a concentration of *myo*-[2-^{3}H]inositol of approx. 80μM). Assuming that the affinity for *myo*-inositol *in vitro* reflects that *in vivo*, then at the concentration of *myo*-inositol found in adult rabbit serum ($77.9 \pm 9.2\mu$M) maximal uptake of *myo*-inositol by adult rabbit lungs would not occur. However, for most of gestation the concentration of *myo*-inositol in foetal rabbit serum is sufficient to saturate the *myo*-inositol uptake system of foetal lung. The developmental decline in serum *myo*-inositol concentration occurs largely after birth in the rabbit, a finding which is consistent with the view that the placenta is a major contributor of *myo*-inositol to the foetal blood. The increased amounts of *myo*-inositol found transiently in neonatal rabbit serum may originate from milk since it is known that the milk of several species contains *myo*-inositol in millimolar concentrations (Macy *et al.*, 1950).

When considering the sources of *myo*-inositol for the developing rabbit lung, the data presented here support the proposition that during foetal development there is a decreased contribution from both synthesis *de novo* within the lung and by uptake from the blood. Such a situation is likely to influence phosphatidylinositol metabolism in the developing lung. This is true in spite of the finding that the total *myo*-inositol content of foetal lung tissue is similar to that of adult rabbit lung tissue and exceeds by far the amount required *in vitro* to sustain maximal phosphatidylinositol synthesis by lung microsomes (Bleasdale *et al.*, 1979). There is evidence that the total *myo*-inositol content of tissues is a poor indicator of *myo*-inositol availability for phosphatidylinositol biosynthesis. First, in several tissues the metabolism of phosphatidylinositol *in vitro* is influenced by the concentration of *myo*-inositol in the extracellular medium even though these tissues contain large amounts of *myo*-inositol (Freinkel *et al.*, 1975; Eichberg *et al.*, 1979). Second, even though adult rabbit lung contains *myo*-inositol in amounts greater than that required for maximal phosphatidylinositol synthesis *in vitro*, the administration *in vivo* of large amounts of *myo*-inositol to adult rabbits was found to increase the phosphatidylinositol content and decrease the phosphatidylglycerol content of their lung surfactant (Hallman & Epstein, 1980). Therefore, the developmental change from a lung surfactant rich in phosphatidylinositol to one rich in phosphatidylglycerol that occurs in the rabbit at the time of birth (Hallman & Gluck, 1980) may be influenced markedly by the decreased availability of *myo*-inositol for the lungs at this time of development. In the human, the production of lung surfactant rich in phosphatidylglycerol commences in the foetus late in gestation rather than at birth as in the rabbit (Hallman *et al.*, 1976). If *myo*-inositol availability is an important factor in the production of phosphatidylglycerol-rich surfactant in the human, then the developmental decline in *myo*-inositol availability should be detectable before term. Campling & Nixon (1954) observed that the concentration of *myo*-inositol in human foetal blood was lower at term than during the first and second trimesters, but no measurements during the third trimester were reported.

Finally, one may speculate that *myo*-inositol may be involved in the delayed appearance of phosphatidylglycerol in the lung surfactant of the human foetus in some pregnancies complicated by diabetes mellitus (Cunningham *et al.*, 1978). Patients with diabetes mellitus are known to suffer an imbalance in *myo*-inositol homeostasis (Clements & Reynertson, 1977) and this has been implicated in the etiology of diabetic neuropathy (Greene *et al.*, 1975). The abnormally high concentrations of *myo*-inositol in the plasma of diabetic patients are corrected by insulin therapy (Clements & Reynertson, 1977). In the pregnant diabetic woman it is possible that an imbalance in *myo*-inositol homeostasis in the mother affects adversely *myo*-inositol homeostasis in the foetus. This could involve increased placental transfer of *myo*-inositol (as a result of abnormally high concentrations of *myo*-inositol in the maternal blood) and/or increased synthesis *de novo* by the placenta and foetus (supported by increased availability of glucose to the foetus). The potential for placental transfer and placental synthesis of *myo*-inositol in the human remains to be determined.

The results of this investigation of *myo*-inositol

availability for the developing rabbit lung together with the earlier report of an effect of *myo*-inositol on rabbit lung surfactant composition (Hallman & Epstein, 1980) support the hypothesis of an involvement of *myo*-inositol in the regulation of the phosphatidylglycerol content of lung surfactant and justify an examination of *myo*-inositol homeostasis in the human foetus.

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