# Mechanisms of hepatic phosphatidylcholine synthesis in the developing guinea pig: contributions of acyl remodelling and of *N*-methylation of phosphatidylethanolamine

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Hepatic phosphatidylcholine (PC) from the immature fetal guinea pig at day 55 of gestation comprised mainly unsaturated molecular species containing $C_{18:2(n-6)}$ and $C_{22:6(n-3)}$ at the *sn*-2 position, reflecting placental permeability to essential fatty acids. At both day 55 and term (day 68), [Me-$^{14}$C]choline was incorporated *in utero* over 3 h largely into *sn*-1-$C_{16:0}$ PC species, with incorporation into *sn*-1-$C_{18:0}$ PC species increasing by 18 h of incubation. Comparison of specific radioactivities after 3 h and 18 h suggests PC acyl remodelling by phospholipase $A_1$. No incorporation into $C_{20:4(n-6)}$-containing PC species could be detected of either [*Me*-$^{14}$C]choline *in vivo* or CDP-[*Me*-$^{14}$C]-choline in isolated microsomes. The major phosphatidylethanolamine (PE) species were 16:0/22:6 and 18:0/22:6. Although [$^{14}$C]ethanolamine was initially incorporated mainly into *sn*-1-$C_{16:0}$ species, specific-radioactivity analysis suggested differential turnover rather than acyl remodelling. [1,2-$^{14}$C]-Ethanolamine and [*Me*-$^{14}$C]methionine incorporation into PC molecular species indicated that both newly synthesized and total PE pools were available for *N*-methylation. Since the PC pool synthesized from PE included $C_{20:4}$- and $C_{22:6}$-containing species, *N*-methylation may provide a mechanism for supplying essential long-chain fatty acids to developing tissues that can be regulated independently from bulk PC synthesis.

## INTRODUCTION

The pathways and mechanisms regulating phosphatidylcholine (PC) synthesis in rat liver have been studied extensively (for review see [1,2]). In addition to direct synthesis *de novo* from diacylglycerol and CDP-choline [3], PC can also be formed by *N*-methylation of phosphatidylethanolamine (PE) [4,5], and modified by acyl remodelling mechanisms [6,7]. The relative fluxes through the CDP-choline and *N*-methylation pathways for PC synthesis are dependent on the availability of dietary choline [8,9] and hormonal balance [10] of the animal. Acyl remodelling involves the sequential actions of phospholipase(s) and acyltransferase(s), and recent evidence has implicated activities of phospholipase $A_1$ ($PLA_1$) and phospholipase $A_2$ ($PLA_2$) in PC synthesis by isolated rat hepatocytes [11]. The final PC composition of specific liver cell membranes is determined by the balance between these various pathways and intracellular phospholipid transport mechanisms. For instance, it has been suggested that *N*-methylation of PE serves specifically to synthesize PC species containing long-chain polyunsaturated fatty acids such as arachidonate [$C_{20:4(n-6)}$] and docosahexaenoate [$C_{22:6(n-3)}$] [12,13]. Indeed, Samborski and his colleagues recently demonstrated preferential conversion of newly synthesized PE 16:0/22:6 to PC 16:0/22:6 (i.e. PE and PC containing the given acyl species) in rat hepatocytes, and presented evidence that PE molecular species are not subject to significant acyl remodelling [14].

One major role for liver PC synthesis is in the assembly and secretion of plasma lipoproteins, both very low density and high density. Continued PC synthesis is essential for hepatic very-low density lipoprotein secretion [15,16], and the choline-deficient rat is characterized by hepatic lipid accumulation [17]. Neutral phospholipids, principally PC, stabilize the lipoprotein particles [18], and may also function in the directed transport of specific fatty acids to selected target tissues [19]. This role may be particularly important for the neonatal rat in the postnatal period of accelerated brain development, characterized by the accumulation of $C_{22:6(n-3)}$ and $C_{20:4(n-6)}$ in grey matter [20].

Although PC synthesis by the CDP-choline and PE *N*-methylation pathways in guinea-pig liver has been well described [21], the relative contribution and specificity of fatty acyl remodelling mechanism(s) have not been studied. There are good reasons for expecting that these may differ substantially from those described in the rat. The fatty acid composition of adult guinea-pig liver PC is very different from that of adult rat liver [22]; in particular, $C_{20:4(n-6)}$ is a minor component of guinea-pig liver PC but the major unsaturated fatty acyl component of rat liver. Additionally, as brain development is largely prenatal in both the guinea pig [23] and human [24], the perinatal guinea pig may prove to be a more suitable animal model than the neonatal rat to study the specificity of PC synthesis in human liver development. The bulk of $C_{22:6}$ acquisition by the developing guinea-pig brain occurs *in utero*, rather than postnatally, and the effect of this demand for $C_{22:6}$ supply by the guinea-pig fetus on the developmental profile of guinea-pig liver phospholipid metabolism is not presently known.

In this study we have applied h.p.l.c. techniques for the analysis of individual molecular species of PC and PE to determine the developmental regulations of their syntheses in perinatal guinea-pig liver, both at the period of maximal brain growth (day 55) [23] and at term (day 68). The results suggest a central role for PC acyl remodelling by a $PLA_1$ rather than a $PLA_2$ mechanism. They also suggest that, despite the considerable differences in composition and developmental changes in guinea-pig liver PC species compared with the rat, many of the

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; $PLA_1$, phospholipase $A_1$; $PLA_2$, phospholipase $A_2$.

fundamental mechanisms regulating the synthesis of PC species containing unsaturated fatty acids may be remarkably similar.

## EXPERIMENTAL

### Materials

[*Me*-$^{14}$C]Choline chloride (55 mCi/mmol), ammonium CDP-[*Me*-$^{14}$C]choline (57 mCi/mmol), L-[*Me*-$^{14}$C]methionine (56 mCi/mmol) and L-3-phosphatidyl[*N*-*Me*-$^{3}$H]choline 1,2 dipalmitoyl (81 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). [1,2-$^{14}$C]Ethanolamine (2.4 mCi/mmol) was from ICN Biomedicals (High Wycombe, Bucks., U.K.). Choline chloride and chloroform were purchased from Merck (Poole, Dorset, U.K.). H.p.l.c.-grade methanol and trifluoroethanol were bought from Rathburn (Walkerburn, Scotland, U.K.). Other chemicals, with noted exceptions, were from Sigma (Poole, Dorset, U.K.).

### Radiolabelling protocols

Dunkin–Hartley guinea-pig fetuses at gestational ages day 55 and 68 (term) were injected intraperitoneally *in utero* with 50 $\mu$Ci of [$^{14}$C]choline or 50 $\mu$Ci of [$^{14}$C]ethanolamine in 0.9% (w/v) NaCl (final volume 0.75 ml). A mid-ventral incision was made in the mother under light halothane/nitrous oxide anaesthesia, and selected fetuses were injected *in situ*. The incision was closed and the mother allowed to recover. At 3 h after the injection, the pups were delivered by Caesarian section and killed by intraperitoneal injection of pentobarbitone. Alternatively, pups at day 68 of gestation were delivered by Caesarian section and immediately injected intraperitoneally with 50 $\mu$Ci of [$^{14}$C]choline, [$^{14}$C]-ethanolamine or [$^{14}$C]methionine, and allowed free access to a lactating surrogate mother and water during the labelling period. Pups that received [$^{14}$C]choline or [$^{14}$C]ethanolamine were killed after 18 h, and pups given [$^{14}$C]methionine were killed after 3 h.

After being blanched with heparinized saline (1 i.u./ml), liver samples were frozen in liquid $N_2$ and the remaining tissue was used in microsomal preparations. Liver was homogenized in buffer [50 mM Tris/HCl, pH 7.4, 0.14 mM NaCl, 5 mM EDTA (disodium salt), 0.2 mM phenylmethanesulphonyl fluoride] at 4 °C. After centrifugation at 10000 *g* for 10 min at 4 °C, microsomes were pelleted at 100000 *g* for 1 h, resuspended in 400 $\mu$l of buffer and stored at −20 °C. Microsomal PC was radiolabelled *in vitro* by incubation of microsomal preparations (100 $\mu$l) from unlabelled fetuses with 0.15 $\mu$Ci of CDP-[$^{14}$C]choline and 20 mM $MgCl_2$ at 37 °C for 3 h.

### Phospholipid analysis

Liver (100–200 mg) homogenized in 0.8 ml of 0.9% (w/v) NaCl or microsomes (100 $\mu$l) were extracted with chloroform and methanol [25]. PC 14:0/14:0 or PE 14:0/14:0 in trifluoroethanol (100 nmol each) was added as internal standard together with $^{3}$H-labelled PC 16:0/16:0 (20 nCi). PC and PE were isolated from the lipid extract on a 100 mg Varian BondElut $NH_2$ cartridge (Jones Chromatography, Hengoed, Glamorgan, U.K.) [26]. Alternatively, in the study of *N*-methylation of PE, PC and PE were isolated by normal-phase h.p.l.c. on a 25 cm aminopropylsilica column (Jones Chromatography). PC and PE fractions were resolved at 50 °C with an isocratic mobile phase of acetonitrile/methanol/15 mM $NH_4H_2PO_4$, $Na_2HPO_4$, pH 5.5 (380:186:25, by vol) at 1 ml/min.

PC and PE molecular species were resolved on an Apex II ODS column (25 cm × 4.6 mm internal diameter) (Jones Chromatography) at 50 °C using a mobile phase of methanol/water (37:3, v/v) containing 40 mM choline chloride at a flow rate of 1 ml/min. This system provided efficient resolution of disaturated as well as unsaturated species [27]. To study *N*-methylation of PE, enhanced resolution of polyunsaturated species of PC or PE was achieved by separating PC or PE on two Apex II ODS columns in series at room temperature, with a mobile phase of methanol/acetonitrile/water (36:3:1, by vol.) containing 40 mM choline chloride at a flow rate of 1 ml/min.

Phospholipids eluted from the h.p.l.c. were quantified on-line by post-column fluorescence derivatization with 1,6-diphenyl-1,3,5-hexatriene [27]. Routine confirmation of species identity was provided by comparison of their fluorescence signal with the u.v. absorbance at 205 nm due to unsaturated acyl groups [27]. The identities of resolved species were determined by g.c. analysis of methyl fatty acids after transmethylation with sodium methoxide [28]. Methyl fatty acid esters were resolved on a fused silica capillary column (30 m × 0.25 mm; DB225; Jones Chromatography), with positional assignment given after hydrolysis with $PLA_2$ [29]. Radioactivity of eluted phospholipid peaks was determined by a dual-channel on-line radiochemical monitor (LB 505) equipped with a solid scintillation flow cell (Berthold Instruments).

## RESULTS

### PC species composition of guinea-pig liver microsomes

The PC species composition of guinea-pig liver microsomes is illustrated by the h.p.l.c. profile in Figure 1; the identities of the major species present are given in Table 1. The ten species identified consistently represented over 90% of total PC. Unlike rat liver PC, which is characterized by a high content of PC 18:0/20:4 [14], the predominant PC species present in fetal and maternal guinea-pig liver were PC 16:0/18:2 and PC 18:0/18:2, and arachidonoyl-containing PC species were minor components. Furthermore, and again unlike the developing rat, there were no dramatic changes in liver microsomal PC species in the developing guinea pig between days 55 of gestation and the adult. The

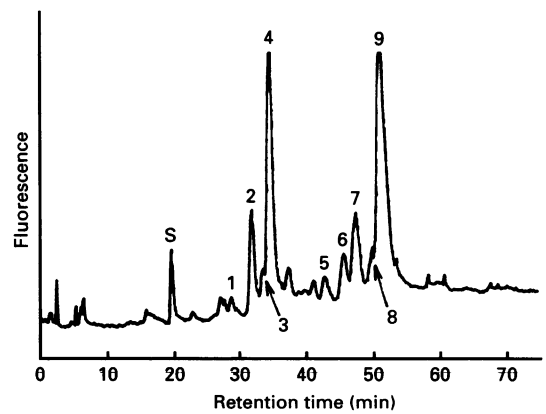


**Figure 1 H.p.l.c. profile of neonatal (day 68) guinea-pig liver microsomal PC**

PC was prepared from chloroform/methanol (1:1, v/v) extracts of guinea-pig liver microsomes on a $NH_2$ BondElut cartridge. Individual PC molecular species 1–9 (see Table 1) were resolved by a single Apex II ODS reversed phase column and quantified relative to the internal standard PC 14:0/14:0 (S) by fluorescence at 460 nm as described in the Experimental section. Peak number 10 (PC 16:0/18:0) has not been indicated because this molecular species was absent from neonatal (day 68) guinea-pig liver microsomes. The fractional distributions of individual molecular species were calculated relative to the total of PC species, including unidentified minor components.

**Table 1 PC molecular species composition of fetal and neonatal guinea-pig liver**

Fetal samples were analysed at days 55 and 68 of gestation, and maternal samples at day 68 of gestation. Results are expressed as percentages of total PC (mean ± S.D.) for whole liver and for isolated microsomal proparations. ND, not detected.

| | PC concentration (% of total) | | | | | |
|---|---|---|---|---|---|---|
| | Fetal (day 55) | | Neonatal (day 68) | | Maternal (day 68) | |
| Molecular species | Liver ($n = 5$) | Microsome ($n = 7$) | Liver ($n = 10$) | Microsome ($n = 9$) | Liver ($n = 3$) | Microsome ($n = 3$) |
| 1. 16:0/18:3 | 3.8 ± 1.3 | 1.7 ± 0.3 | 2.1 ± 0.6 | 1.6 ± 0.8 | 1.4 ± 0.6 | 1.5 ± 1.1 |
| 2. 16:0/22:6 | 10.3 ± 2.8 | 7.4 ± 1.0 | 9.1 ± 2.0 | 9.7 ± 1.3 | 6.4 ± 0.4 | 7.0 ± 1.3 |
| 3. 16:0/20:4 | 3.4 ± 0.9 | 2.3 ± 0.4 | 3.6 ± 0.7 | 3.6 ± 1.0 | 2.4 ± 0.3 | 2.4 ± 0.8 |
| 4. 16:0/18:2 | 17.4 ± 1.2 | 18.3 ± 3.6 | 28.8 ± 2.8 | 26.6 ± 9.4 | 26.4 ± 0.9 | 26.6 ± 3.2 |
| 5. 16:0/16:0 | 8.2 ± 0.6 | 12.3 ± 2.2 | 3.8 ± 1.8 | 5.0 ± 5.0 | 2.9 ± 0.9 | 3.4 ± 0.4 |
| 6. 16:0/18:1 | 9.4 ± 2.7 | 6.8 ± 2.5 | 5.9 ± 1.7 | 7.0 ± 1.8 | 6.5 ± 0.7 | 7.4 ± 0.8 |
| 7. 18:0/22:6 | 11.8 ± 3.2 | 12.1 ± 4.2 | 9.4 ± 2.2 | 8.1 ± 2.8 | 7.5 ± 1.2 | 11.3 ± 1.8 |
| 8. 18:0/20:4 | 5.4 ± 1.0 | 4.7 ± 1.0 | 3.4 ± 1.2 | 3.1 ± 1.1 | 2.3 ± 0.7 | 2.6 ± 2.0 |
| 9. 18:0/18:2 | 22.7 ± 2.2 | 29.1 ± 1.6 | 30.1 ± 1.9 | 30.8 ± 4.3 | 38.9 ± 1.1 | 38.0 ± 1.9 |
| 10. 16:0/18:0 | 7.1 ± 2.9 | 5.0 ± 1.5 | 2.2 ± 1.4 | ND | 4.8 ± 2.1 | ND |

**Table 2 Incorporation of [$^{14}$C]choline into guinea-pig liver microsomes *in vivo* over 3 h**

Results are presented as mean ± S.D. of total incorporation. Also shown is the ratio (R/M ratio) of these values to the percentage distribution of PC mass described in Table 1. ND, not determined.

| | [$^{14}$C]Choline incorporation into microsomal PC | | | | | |
|---|---|---|---|---|---|---|
| | Fetal (day 55, $n = 3$) | | Neonatal (day 68, $n = 3$) | | Maternal (day 68, $n = 3$) | |
| Molecular species | (% of total) | (R/M ratio) | (% of total) | (R/M ratio) | (% of total | (R/M ratio) |
| 16:0/18:3 | 5.6 ± 3.8 | 3.29 | 7.2 ± 2.6 | 4.51 | 3.0 ± 0.2 | 2.0 |
| 16:0/22:6 | 15.8 ± 2.6 | 2.14 | 17.6 ± 1.6 | 1.81 | 11.0 ± 0.4 | 1.57 |
| 16:0/20:4 | ND | | ND | | ND | |
| 16:0/18:2 | 33.9 ± 1.6 | 1.95 | 41.5 ± 0.3 | 1.56 | 49.6 ± 1.8 | 1.86 |
| 16:0/16:0 | 11.5 ± 0.8 | 1.41 | 4.0 ± 0.4 | 0.80 | 5.0 ± 0.3 | 1.47 |
| 16:0/18:1 | 12.2 ± 1.1 | 1.29 | 10.4 ± 0.8 | 1.49 | 5.7 ± 0.2 | 0.77 |
| 18:0/22:6 | 7.4 ± 0.8 | 0.63 | 6.9 ± 1.2 | 0.85 | 11.6 ± 0.9 | 1.03 |
| 18:0/20:4 | ND | | ND | | ND | |
| 18:0/18:2 | 13.6 ± 0.5 | 0.60 | 12.3 ± 1.2 | 0.40 | 14.3 ± 0.3 | 0.38 |
| 16:0/18:0 | ND | | ND | | ND | |

chromatogram in Figure 1 was from a newborn term pup (day 68 of gestation), but was essentially no different from that of the maternal guinea pig. This comparison is shown in detail in Table 1, which also demonstrates that there were few differences in PC composition between whole liver tissue and liver microsomes. Between day 55 and day 68 of gestation there were small, but significant ($P < 0.05$), reciprocal decreases in the fractional concentrations of the saturated species PC 16:0/16:0 and PC 16:0/18:0 and increases in the unsaturated species PC 16:0/18:2 and PC 18:0/18:2.

## Incorporation of [$^{14}$C]choline into liver PC

The fractional distribution of the incorporation of [$^{14}$C]choline over 3 h *in utero* into individual PC molecular species of liver microsomes is detailed in Table 2. The incorporation patterns at the same time into total liver PC were essentially identical with the microsomal distribution at both day 55 and day 68 of gestation. Although [$^{14}$C]choline was incorporated into generally similar species, there were striking differences in the distribution of radioactivity (Table 2) compared with that of PC mass (Table 1). Most significantly, [$^{14}$C]choline was incorporated principally into PC species with palmitoyl at the *sn*-1 position. Although at both gestational ages, PC 18:0/18:2 was the major species present [29.1% and 30.8% at day 55 and day 68 respectively (Table 1)], [$^{14}$C]choline incorporation was greatest into PC 16:0/18:2 [33.9 and 41.5% respectively at day 55 and day 68 of gestation (Table 2)]. Comparison of distribution patterns of [$^{14}$C]choline into PC with PC mass (Table 2; radioactivity/mass ratio) strongly suggests that, in common with adult rat hepatocytes [11], the predominant PC acyl remodelling mechanism active in guinea-pig liver involves a $PLA_1$ activity converting PC species synthesized initially with *sn*-1-palmitoyl into species with *sn*-1 stearoyl. Inspection of the incorporation of [$^{14}$C]choline into maternal guinea-pig liver PC species (Table 2) showed identical synthetic mechanisms in the adult animal. The involvement of $PLA_1$ was supported by the distribution of [$^{14}$C]choline incorporation into liver PC of term guinea pigs over a more prolonged time (Table 3). Pups were injected with [$^{14}$C]choline at delivery, and killed 18 later. Although there were no significant differences in PC composition over this period (compare Tables 1 and 3), the [$^{14}$C]choline incorporation pattern

**Table 3 Dynamics of PC acyl remodelling in liver microsomes of term neonatal guinea pigs**

Guinea-pig pups at day 68 of gestation were injected with 50 $\mu$Ci of [$^{14}$C]choline either for 3 h before delivery by Caesarian section or for 18 h postnatally. Liver microsomal preparations from additional day 68 guinea-pig pups were made both at delivery and 18 h postnatally, and were used for the *in vitro* incorporation of CDP-[$^{14}$C]choline into PC molecular species. Results (mean ± S.D.) are presented as % of fractional incorporations into PC of [$^{14}$C]choline *in vivo* and CDP-[$^{14}$C]choline *in vitro*, and as specific radioactivity (d.p.m./nmol) of the [$^{14}$C]choline incorporation *in vivo*. Total incorporations of [$^{14}$C]choline into PC *in vivo* at 3 h and 18 h were respectively $(2.46 \pm 0.81) \times 10^5$ and $(3.04 \pm 0.99) \times 10^5$ d.p.m./g of liver (39 ± 3 and 46 ± 7 d.p.m./nmol of PC). ND, not determined.

| | [$^{14}$C]Choline incorporation in neonatal guinea-pig liver (18 h postnatal) | | | Specific radioactivity of PC after [$^{14}$C]choline incorporation *in vivo* | | Incorporation *in vitro* of CDP-[$^{14}$C]choline into microsomal PC | |
|---|---|---|---|---|---|---|---|
| Molecular species | Tissue (% of total PC) ($n = 3$) | Microsomes (% of total PC) ($n = 3$) | [$^{14}$C]choline to PC *in vivo* ($n = 3$) | *In utero*, 3 h ($n = 3$) | Postnatal, 18 h ($n = 3$) | *In utero*, 3 h ($n = 3$) | Postnatal, 18 h ($n = 2$) |
| 16:0/18:3 | 2.7 ± 2.4 | 1.3 ± 0.5 | 8.0 ± 0.5 | 1291 ± 381 | 1353 ± 767 | 7.4 ± 1.6 | 12.8 |
| 16:0/22:6 | 8.4 ± 0.6 | 8.2 ± 0.9 | 9.4 ± 1.4 | 240 ± 83 | 321 ± 68 | 14.1 ± 1.8 | 11.3 |
| 16:0/20:4 | 3.0 ± 0.5 | 3.4 ± 0.6 | ND | ND | ND | ND | ND |
| 16:0/18:2 | 17.2 ± 1.8 | 23.8 ± 3.7 | 23.4 ± 1.4 | 179 ± 52 | 244 ± 60 | 46.6 ± 3.7 | 36.7 |
| 16:0/16:0 | 4.5 ± 0.5 | 3.9 ± 1.2 | 5.0 ± 0.4 | 415 ± 195 | 223 ± 251 | 6.1 ± 0.9 | 9.2 |
| 16:0/18:1 | 8.5 ± 0.4 | 7.9 ± 0.4 | 8.2 ± 0.4 | 195 ± 43 | 123 ± 24 | 7.5 ± 1.6 | 9.0 |
| 18:0/22:6 | 12.4 ± 1.3 | 12.9 ± 3.0 | 9.3 ± 1.0 | 96 ± 11 | 212 ± 79 | 6.2 ± 0.1 | 5.2 |
| 18:0/20:4 | 6.3 ± 1.1 | 5.0 ± 0.9 | ND | ND | ND | ND | ND |
| 18:0/18:2 | 29.4 ± 0.1 | 35.5 ± 1.8 | 36.0 ± 0.8 | 47 ± 12 | 227 ± 23 | 13.3 ± 3.1 | 15.6 |
| 16:0/18:0 | 7.2 ± 1.3 | ND | ND | ND | ND | ND | ND |

over 18 h differed radically from that over 3 h, with the fractional incorporation into PC 18:0/18:2 increasing 3-fold and that into PC 16:0/18:2 showing a reciprocal decline. This difference was not due to any postnatal change in the regulation of PC metabolism, as essentially identical incorporation patterns were seen over a separate 24 h incorporation protocol *in utero* at day 68 of gestation (results not shown).

It is possible that the apparent redistribution of [$^{14}$C]choline incorporation between 3 h and 18 h of incubation may have been a consequence of selective catabolism of *sn*-1-palmitoyl species, with little parallel remodelling to the *sn*-1-stearoyl species. Whereas unequivocal determination of the relative significances of these alternative mechanisms requires a pulse–chase protocol [11] that is not feasible *in vivo*, calculations of specific radioactivities of individual molecular species of PC at both 3 h and 18 h supports the concept of *sn*-1 acyl remodelling (Table 3). Total incorporation of [$^{14}$C]choline into PC was very similar at both time points (39 compared with 46 d.p.m./nmol of PC), presumably reflecting the net balance *in vivo* between synthesis *de novo* and lipoprotein secretion and PC catabolism. There were highly significant increases in the specific radioactivities of the major *sn*-1-stearoyl species, PC 18:0/18:2 and PC 18:0/22:6. The specific radioactivities of PC 16:0/18:2 and PC 16:0/22:6 also increased over this time period, but the increase was much smaller than that of the *sn*-1-stearoyl species and was expected because of the continued incorporation *de novo* of [$^{14}$C]choline. Interestingly, with respect to the supply of $n-3$ fatty acids, the specific radioactivity of PC 16:0/18:3 was very high at both time points despite being a relatively minor component in terms of PC mass. It is possible, however, that PC 16:0/18:3 may have included other molecular species which could have contributed to the amount of radiolabel apparently associated with this species.

### Microsomal incorporation of CDP-[$^{14}$C]choline into PC *in vitro*

Further support for the concept of acyl remodelling by $PLA_1$ was provided by the incorporation *in vitro* by isolated liver microsomes of CDP-[$^{14}$C]choline into PC. At day 68 of gestation, the incorporation patterns of CDP-[$^{14}$C]choline into individual molecular species of microsome PC over 3 h *in vitro* were essentially identical (Table 3) with that previously described for [$^{14}$C]choline into fetal guinea-pig liver microsomal PC at the same gestational age *in utero* (Table 2). Again there was predominant incorporation of radiolabel into PC 16:0/18:2, with that into PC 18:0/18:2 being considerably lower. This same ratio between PC 16:0/18:2 and PC 18:0/18:2 was maintained for CDP-[$^{14}$C]choline incorporation into PC over 3 h by microsomes isolated from neonatal guinea-pig liver at 18 h postnatal age (Table 3). The incorporated CDP-[$^{14}$C]choline was not subjected to significant acyl remodelling under the incubation conditions employed *in vitro*, as the distribution pattern of radiolabel into microsomal PC species was not altered by prolonged incubation up to 10 h at 37 °C (results not shown).

### Synthesis of PC containing long-chain polyunsaturated fatty acids

PC acyl remodelling mediated by a $PLA_1$ activity can readily explain the patterns of synthesis of the major unsaturated PC species of guinea-pig liver, PC 18:0/18:2, PC 16:0/18:2, PC 16:0/22:6 and PC 18:0/22:6. Inspection of the patterns of synthesis of other unsaturated PC species, however, suggests that additional mechanisms must also act to determine the molecular specificity of PC synthesis. First, there was considerable synthesis *de novo* of PC 16:0/18:3 both *in vivo* (Table 2) and *in vitro* (Table 3), but this species remained a minor component of microsomal and total liver PC (Table 1). Newly synthesized PC 16:0/18:3 was not converted into PC 18:0/18:3, as this species was not detected either in mass terms or in the 18 h [$^{14}$C]choline incorporation protocol. Using our h.p.l.c. protocol, PC 18:0/18:3 would have been eluted slightly in advance of PC 16:0/16:0. Secondly, although PC 16:0/20:4 and PC 18:0/20:4 were minor components of total PC, they were consistently present in measurable amounts. At no gestational age, either *in vivo* or *in vitro*, could any incorporation of radioactivity above background rates be detected into either species. As *N*-methylation of PE has been described as one mechanism for the supply of unsaturated PC species [30], the specificity of this alternative pathway for the

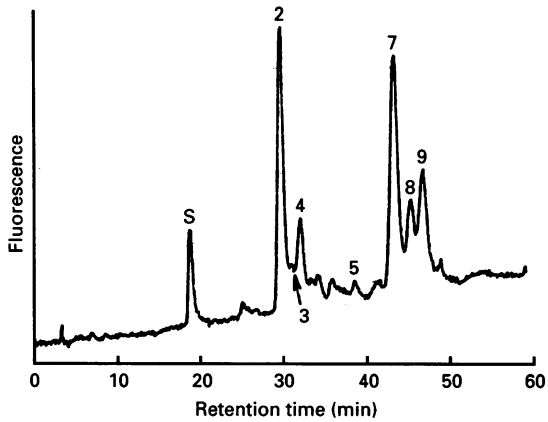


**Figure 2 H.p.l.c. profile of neonatal (day 68) guinea-pig liver PE**

Guinea-pig liver PE was isolated from chloroform/methanol extract by $NH_2$ BondElut cartridge. Individual molecular species 2–5 and 7–9, identified in Table 4, were resolved by a single Apex II ODS reversed-phase column and quantified by fluorescence as described in the Experimental section. S indicates the position of the internal standard PE 14:0/14:0.

synthesis of PC was then studied in term neonatal guinea-pig liver.

### Incorporation of [$^{14}$C]ethanolamine into liver PE

The molecular species composition of whole liver PE from term neonatal guinea pigs is shown in Figure 2 and summarized in Table 4. The composition of PE in whole liver and liver microsomes was the same at days 55 and 68 of gestation and in adult maternal liver (result not shown), and differed fundamentally from that of PC from the same tissue fractions. PE 16:0/22:6 and PE 18:0/22:6 together comprised over 50% of total PE species, with the other major components being PE 18:0/20:4 and PE 18:0/18:2. Over a 3 h period *in utero*, [$^{14}$C]ethanolamine was incorporated into the same species, but with a greatly different distribution pattern (Table 4). There was a preponderance of incorporation into *sn*-1-palmitoyl rather than *sn*-1-stearoyl species, with PE 16:0/22:6 accounting for more than 40% of the total. Over 18 h with [$^{14}$C]ethanolamine *in vivo* in neonatal guinea pigs, the distribution of $^{14}$C more closely resembled that of PE mass (Table 4). We have no evidence, however, that this redistribution of radioactivity represented remodelling at the *sn*-1 position. Unlike the incorporation of [$^{14}$C]choline into total PC (Table 2), that of [$^{14}$C]ethanolamine into total PE decreased rather than increased between the 3 h and 18 h incubation periods, and there were no increases in specific radioactivities of any PE species (Table 4). This observation is consistent with preferential mobilization or catabolism of *sn*-1-palmitoyl PE species, without substantial reacylation to *sn*-1-stearoyl species. One additional striking difference between the synthetic patterns of PC and PE species was the appreciable incorporation of [$^{14}$C]ethanolamine into both PE 16:0/20:4 and PE 18:0/20:4 species (Table 4), compared with the absence of detectable [$^{14}$C]choline incorporation into equivalent PC species (Table 2).

### *N*-Methylation of PE

The molecular species distribution of incorporation of [$^{14}$C]ethanolamine into liver PC of the term guinea pig is given in Table 4. This distribution for 3 h incubation with radiolabel *in utero* was identical with the comparable incorporation of [$^{14}$C]ethanolamine into PE, with PC 16:0/22:6 and then PC 18:0/22:6 being most heavily labelled. Over 18 h postnatally, however, the distribution pattern was very different. Radiolabel was lost from 22:6-containing species and accumulated principally in PC 18:0/18:2. This pattern resembled the composition of PC more closely than that of PE, suggesting acyl remodelling after *N*-methylation. The suggestion that only newly synthesized PE is substrate for *N*-methylation [31] was not supported by results of the incorporation of [$^{14}$C]methionine into liver PC species over 3 h in neonatal guinea pigs (Table 4). The distribution of [$^{14}$C]methionine into PC species was identical with the total PE fractional concentration distribution at the same time (Table 4), suggesting no selectivity for bulk *N*-methylation *in vivo*.

## DISCUSSION

Recent studies have described detailed mechanisms for the regulation of liver PC synthesis in the adult rat [9,11,30,31,32]. The extent to which such mechanisms are applicable to other animal species is uncertain, with any diversity in regulatory mechanisms of PC synthesis being expected to be most apparent in the fetal and neonatal periods. In this context, the guinea pig

**Table 4 PE molecular species composition, synthesis and *N*-methylation to PC in liver from neonatal (day 68) guinea pigs *in vivo***

Pups were injected at Caesarian section delivery with 50 $\mu$Ci of [$^{14}$C]ethanolamine, and were killed and their livers removed at either 3 h or 18 h postnatally. Alternatively, pups were injected with 50 $\mu$Ci of [$^{14}$C]methionine, and were killed and their livers removed after 3 h. Results (mean ± S.D.) are presented as % of PE mass composition, the % distribution of [$^{14}$C]ethanolamine incorporation into either PE or PC, the specific radioactivity (d.p.m./nmol) of the incorporation into PE and the % distribution of [$^{14}$C]methionine into PC by *N*-methylation of PE. At 3 h and 18 h postnatally total liver PE concentrations were respectively 5.3 ± 0.5 and 7.1 ± 1.5 $\mu$mol/g of liver, incorporations into PE were 11.36(± 8.85) × $10^4$ and 1.81(± 0.59) × $10^4$ d.p.m./g of liver and into PC were (6.01 ± 3.86) × $10^4$ and (2.20 ± 0.72) × $10^4$ d.p.m./g of liver. ND, not determined.

| Molecular species | PE composition (% of total) | | [$^{14}$C]Ethanolamine incorporation (% of total) | | | | PE specific radioactivity (d.p.m./nmol) | | [$^{14}$C]Methionine incorporation into PC (% of total) ($n$ = 3) |
|---|---|---|---|---|---|---|---|---|---|
| | | | 3 h ($n$ = 3) | | 18 h ($n$ = 3) | | | | |
| | 3 h | 18 h | PE | PC | PE | PC | 3 h | 18 h | |
| 2. 16:0/22:6 | 21.4 ± 1.8 | 25.2 ± 3.4 | 44.0 ± 2.0 | 38.5 ± 1.4 | 20.4 ± 2.1 | 11.7 ± 1.0 | 6.1 ± 0.8 | 1.0 ± 0.5 | 21.3 ± 1.1 |
| 3. 16:0/20:4 | 2.7 ± 1.0 | 3.4 ± 0.9 | 9.2 ± 0.9 | 9.6 ± 1.6 | 18.0 ± 2.1 | 5.6 ± 2.4 | 9.5 ± 2.1 | 2.3 ± 0.3 | 8.5 ± 1.4 |
| 4. 16:0/18:2 | 8.1 ± 1.2 | 3.2 ± 0.8 | 13.6 ± 0.9 | 14.4 ± 2.5 | 11.7 ± 0.6 | 19.2 ± 0.6 | 5.1 ± 1.6 | 3.9 ± 0.7 | 9.5 ± 1.4 |
| 5. 16:0/16:0 | 1.2 ± 0.7 | 1.5 ± 0.5 | ND | ND | ND | ND | ND | ND | 0.8 ± 0.3 |
| 7. 18:0/22:6 | 33.0 ± 2.7 | 30.0 ± 6.9 | 18.8 ± 5.4 | 22.3 ± 3.5 | 28.3 ± 2.3 | 16.9 ± 2.1 | 2.6 ± 1.0 | 1.3 ± 0.8 | 29.3 ± 0.8 |
| 8. 18:0/20:4 | 19.0 ± 4.2 | 14.2 ± 2.2 | 4.8 ± 0.8 | 6.9 ± 2.3 | 11.0 ± 1.2 | 13.0 ± 1.1 | 1.0 ± 0.1 | 1.3 ± 0.8 | 16.4 ± 1.6 |
| 9. 18:0/18:2 | 14.6 ± 3.8 | 19.2 ± 3.3 | 6.7 ± 0.3 | 8.2 ± 1.0 | 20.6 ± 0.5 | 33.7 ± 1.8 | 2.2 ± 1.3 | 0.8 ± 0.4 | 13.8 ± 1.4 |

may be a model of the development of hepatic PC metabolism in man. The guinea pig, like man and unlike the rat, has a haemomonochorial placenta which is permeable to and concentrates essential fatty acids [33], it is born with considerable storage lipid reserves [34], and brain development is largely a prenatal, not postnatal, event [35]. The results presented in this paper are, to our knowledge, the first description in terms of the analysis of individual molecular species of the regulation of liver PC metabolism in the fetal, neonatal and adult guinea pig.

Both the differences and similarities between the guinea pig and the rat in their respective regulatory mechanisms of liver PC synthesis are striking. Marked differences were observed in both PC composition and the ontogeny of the development of that composition. First, adult guinea-pig liver PC was composed primarily of PC 16:0/18:2 and PC 18:0/18:2 (Table 1); arachidonate-containing species, which predominate in the rat [36], were present in only trace amounts. This difference was not dietary in origin, as total $n-6$ fatty acids were comparable in the guinea-pig diet (30.3% of total fatty acids supplied; FD1, Special Diets Services, Witham, Essex, U.K.) and the rat diet (37.4%, Rat and Mouse Maintenance Diet no. 1, Special Diets Services). Secondly, there was no significant alteration in maternal liver PC composition or concentration throughout gestation (Table 1), in contrast with the dramatic increase in total PC and PC 16:0/22:6 that occurs in maternal rat liver at term [37]. Thirdly, fetal guinea-pig liver PC composition at term (day 68) closely resembled that of maternal liver PC (Table 1). Whereas there was a gradual maturation of liver PC composition between day 55 of gestation and term, with the fractional contents of PC 16:0/16:0 and PC 16:0/18:1 decreasing, there was no dramatic change in either PC concentration or composition after delivery (Table 1), again in contrast with the rat [38].

However, despite these differences between the guinea pig and rat, many of the mechanisms underlying the regulations of their liver PC syntheses were very similar. The incorporations into guinea-pig liver PC over 3 h at [$^{14}$C]choline *in vivo* (Table 3) or CDP-[$^{14}$C]choline *in vitro* in isolated microsomes (Table 3) demonstrated the preferential synthesis of *sn*-1-palmitoyl species, principally PC 16:0/18:2. Comparison of this incorporation pattern over 3 h with that of [$^{14}$C]choline into liver PC over 18 h *in vivo* (Table 3) strongly suggested acyl remodelling by a $PLA_1$ mechanism. The time scale of this acyl remodelling was relatively slow, as the incorporation patterns over 3 h into PC species of [$^{14}$C]choline *in vivo* and CDP-[$^{14}$C]choline *in vitro* were essentially identical.

Moreover, an identical incorporation pattern was observed over the shortest incubation period with CDP-[$^{14}$C]choline *in vitro* (0.5 h). A comparable $PLA_1$-mediated mechanism has recently been reported in rat liver, using a pulse–chase protocol of [$^{14}$C]choline incorporation into molecular species of PC in choline-depleted hepatocytes [11]. This has been supported by analyses of PC acyl turnover in rat liver using the $^{18}$O technique [39], and of the catabolism of plasma lipoprotein PC 16:0/18:2 [40]. Moreover, in fetal guinea-pig liver, the basis of this $PLA_1$-mediated PC acyl remodelling was already fully established by day 55 of gestation (Table 3). The increased specific radioactivity of [$^{14}$C]choline incorporation into PC 18:0/18:2 between 3 h and 18 h incubation *in vivo* (Table 3) supports the concept of $PLA_1$ remodelling, rather than there being just preferential catabolism of *sn*-1-palmitoyl species. Although no corresponding decrease in the specific radioactivity of PC 16:0/18:2 was observed over the same time period, this was probably due to the continued synthesis *de novo* of this species. The pool size of choline metabolites in guinea-pig liver precluded any possibility of employing an effective pulse–chase protocol *in vivo*.

The emphasis on $PLA_1$-mediated acyl remodelling does not necessarily deny a potential parallel role for a $PLA_2$-mediated mechanism. At no time, however, did any of the results support any conversion of *sn*-2-linoleoyl species into *sn*-2-arachidonoyl species, as proposed by MacDonald and Thompson [21]. At no gestational age or incubation time with [$^{14}$C]choline was resolvable incorporation detected into either PC 16:0/20:4 or PC 18:0/20:4.

In contrast with their different compositions of PC species, guinea-pig liver PE (Table 4) and rat liver PE species compositions [14] were very similar. In addition, the details of *N*-methylation of PE species to PC appeared to be essentially the same between the two animal species. The incorporation of [$^{14}$C]ethanolamine into PE over 3 h *in vivo* at day 68 of gestation was predominantly into PE 16:0/22:6 ($44.0 \pm 2.0$% total, Table 4), even though PE 18:0/22:6 was the major PE species ($33.0 \pm 2.7$%) in concentration terms. Our results do not support any acyl remodelling of *sn*-1-palmitoyl to *sn*-1-stearoyl PE species, as the specific radioactivity of PE 18:0/22:6 did not increase between 3 h and 18 h of incubation with [$^{14}$C]ethanolamine *in vivo* (Table 4). Rather, these results agree with the selective catabolism and turnover of PE 16:0/22:6 proposed by Samborski and colleagues to occur in rat hepatocytes [14]. Also in agreement with this previous report, [$^{14}$C]ethanolamine incorporation into PC species over 3 h *in vivo* reflected the distribution of [$^{14}$C]ethanolamine into PE species (Table 4). Over the 18 h incubation with [$^{14}$C]ethanolamine, distribution of radiolabel in PC reflected that of PC rather than PE mass, except for detectable syntheses of both PC 16:0/20:4 and PC 18:0/20:4. The most straightforward interpretation of these data involves acyl remodelling of PC newly synthesized from PE by both $PLA_1$- and $PLA_2$-mediated mechanisms.

The results of [$^{14}$C]methionine incorporation into PC species, however, do not support the concept of preferential *N*-methylation of newly synthesized PE species to PC [31]. The incorporation pattern of [$^{14}$C]methionine (Table 4) closely reflected the total PE species composition (Table 4), suggesting little inherent substrate selectivity of the *N*-methylation pathway. Possible explanations for the discrepancy between the incorporation patterns of [$^{14}$C]ethanolamine and [$^{14}$C]methionine into PC (Table 4) include multiple substrate pools of PE with distinct metabolic origins, increased substrate cycling of *sn*-1-palmitoyl PE species resulting in preferential labelling of PE 16:0/22:6 with [$^{14}$C]ethanolamine, or rapid transport of PE newly synthesized from [$^{14}$C]ethanolamine away from the endoplasmic reticulum. In the latter possibility, it would appear that only newly synthesized PE was methylated to PC if recycling of PE back to the endoplasmic reticulum was a relatively slow process. Further exploration of these pathways will require pulse–chase studies in isolated hepatocytes. The similarity of the fundamental principles of PC synthesis between rat and guinea-pig livers, irrespective of their considerable differences in detailed compositions, lends significant support to the central roles of $PLA_1$ remodelling mechanisms.

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