Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine biosynthesis using $^{13}$C NMR spectroscopy

Nicholas V. Reo $^{a,b,*}$, Mehdi Adinehzadeh $^{a,1}$, Brent D. Foy $^b$

$^a$ Department of Biochemistry and Molecular Biology, WSU Magnetic Resonance Laboratory, Wright State University, Cox Institute, Dayton, OH 45429, USA

$^b$ Department of Physics, WSU Magnetic Resonance Laboratory, Wright State University, Cox Institute, 3525 Southern Blvd., Dayton, OH 45429, USA

Received 2 May 2001; received in revised form 18 October 2001; accepted 26 November 2001

Abstract

Choline and ethanolamine are substrates for de novo synthesis of phosphatidylcholine (PtdC) and phosphatidylethanolamine (PtdE) through the CDP-choline and CDP-ethanolamine pathways. In liver, PtdE can also be converted to PtdC by PtdE $N$-methyltransferase (PEMT). We investigated these kinetics in rat liver during a 60 min infusion with $^{13}$C-labeled choline and ethanolamine. NMR analyses of liver extracts provided concentrations and $^{13}$C enrichments of phosphocholine (Pcho), phosphoethanolamine (Peth), PtdC, and PtdE. Kinetic models showed that the de novo and PEMT pathways are ‘channeled’ processes. The intermediary metabolites directly derived from exogenous choline and ethanolamine do not completely mix with the intracellular pools, but are preferentially used for phospholipid synthesis. Of the newly synthesized PtdC, about 70% was derived de novo and 30% was by PEMT. PtdC and PtdE de novo syntheses displayed different kinetics. A simple model assuming constant fluxes yielded a modest fit to the data; allowing upregulated fluxes significantly improved the fit. The ethanolamine-to-Peth flux exceeded choline-to-Pcho, and the rate of PtdE synthesis (1.04 mmol/h/g liver) was 2–3 times greater than that of PtdC de novo synthesis. The metabolic pathway information provided by these studies makes the NMR method superior to earlier radioisotope studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Phospholipid biosynthesis; NMR spectroscopy; Phosphatidylcholine; Phosphatidylethanolamine

1. Introduction

Phosphatidylcholine (PtdC) and phosphatidylethanolamine (PtdE) are the two major phospholipid constituents of biological membranes. Biosynthesis and metabolism of these and other phospholipids are important for proliferation of membrane-bound organelles, lipoprotein synthesis, and signal transduction affecting processes of cell proliferation, differentiation, and apoptosis. Yet our understanding of this metabolism and its regulation is far from complete.

In the early 1970s Sundler et al. [1–3] examined the rates of synthesis for liver PtdC and PtdE using radioisotope methods. Their data could not be described by a simple precursor–product relationship and raised questions about compartmentation of metabolite pools and/or channeling of metabolic pathways, which are yet to be fully answered. Additionally,
many questions about the metabolic pathways themselves remain unanswered. For instance, PtdC can be synthesized from choline via the Kennedy pathway (de novo pathway) or through the sequential methylation of PtdE catalyzed by PtdE N-methyltransferase (PEMT). What is the significance and physiological role of these pathways? In recent studies, Vance et al. [4–6] have provided evidence that PtdC synthesized by the two pathways may be functionally different, and the relative activities of these pathways may play a regulatory role in liver carcinogenesis. Recent studies in our laboratory concerning xenobiotic-induced hepatocarcinogenesis have led us to develop NMR methods to study the biosynthesis of PtdC and PtdE in liver in vivo [7]. The development of methods for pathway-specific analyses of phospholipid biosynthesis in intact tissue can aid in our understanding of numerous cellular processes, and may especially be important for studies of liver cancers.

Here we report results of a NMR experiment designed to monitor the synthesis of PtdC and PtdE in rat liver in vivo. Through the use of specific 13C-labeled substrates in conjunction with 13C NMR, we monitored the biosynthesis of PtdC by the de novo and PEMT pathways simultaneously. Measurements of concentrations and 13C enrichments of various precursor metabolites and phospholipid end products provided data for kinetic modeling of the biosynthetic pathways. These studies enabled us to re-evaluate the kinetics of phospholipid biosynthesis in rat liver during infusion with choline and ethanolamine. Additionally, the NMR technique provided a detailed examination of the specific metabolic pathways. Our findings provide compelling evidence that phospholipid biosynthesis involves ‘channeled’ pathways whereby newly synthesized intermediates are preferentially used to form phospholipid products. This corroborates some earlier reports in the literature [1,2] and lends further support for more recent studies which aim to demonstrate channeling in various cell systems [8–11].

2. Materials and methods

2.1. Materials

Carbon-13 enriched (99 atom %) [1-13C]choline and [2-13C]ethanolamine were purchased from Isotec (Miamisburg, OH, USA). Non-labeled choline and ethanolamine were purchased from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade and purchased from standard sources. Male Fischer-344 rats (243–311 g) were obtained from Harlan (St. Louis, MO, USA) and housed at 22°C with a 12 h light/dark cycle. Rats were fed Teklad MRH 22/5 rodent diet No. 8640. All animals were fasted for 17–22 h prior to experiments.

2.2. Experimental strategy

As shown in Fig. 1, the de novo synthesis of PtdC and PtdE from [1-13C]choline and [2-13C]ethanolamine yields C1-PtdC and C2-PtdE, respectively. These are commonly known as the CDP-choline and CDP-ethanolamine pathways, or the Kennedy pathways. We will also refer to this as de novo synthesis. In liver, an alternative pathway for PtdC synthesis involves the triple methylation of PtdE with S-adenosylmethionine as the methyl donor. The enzyme catalyzing this reaction is PEMT, and the PtdC synthesized via this pathway attains 13C labeling from ethanolamine (i.e. C2-PtdC). The 13C enrichment at specific carbon sites in these phospholipids can be measured by 13C NMR. Additionally, 13C labeling of the precursor carbon sites in these phospholipids can also be measured. Thus, one can monitor the kinetics of phospholipid biosynthesis from the de novo and PEMT pathways simultaneously.

2.3. Experimental protocol

An equimolar solution (18 mM) of [1-13C]choline and [2-13C]ethanolamine was prepared in heparinized saline (2.48 U heparin/ml of 0.9% NaCl). Rats were anesthetized with halothane (5% induction and 1% maintenance) and a catheter was inserted into the left femoral vein. A solution of heparinized saline was infused at 0.026 ml/min to maintain the i.v. line patent. Rats were then infused with the 13C-labeled choline/ethanolamine solution at 0.5 μmol/min for 0, 15, 30, 45, and 60 min (n = 4–5 at each time point). The 0 min data provided a baseline time point and were obtained from rats infused only with saline (no substrates). An analogous experiment
was also conducted in which rats \((n = 2)\) were infused with non-labeled choline and ethanolamine for 60 min. These data aided in our identification of \(^{13}\)C spectral assignments for labeled compounds. After the infusion livers were surgically exposed, freeze-clamped between aluminum tongs chilled in liquid N\(_2\), and stored under liquid N\(_2\) for subsequent analyses.

2.4. Liver extracts

Chemical extracts of the tissues were prepared using a dual phase extraction procedure as described by Tyagi et al. [12]. This dual phase procedure enabled simultaneous extraction of both aqueous and lipid fractions. Briefly, livers were homogenized in three stages: first in methanol (MeOH), then MeOH:CDCl\(_3\), and finally MeOH:CDCl\(_3\):H\(_2\)O (1:1:1 v/v/v; 21 ml/g of liver). The homogenate was then centrifuged (2000 \(\times\) g for 25 min) to allow separation into two liquid phases and a pellet. The sample was transferred through filter paper to a separatory funnel and the pellet was washed three times with the extracting solvent system. The solution was left to stand at 4°C for 17–24 h to allow complete separation of the two liquid phases. The upper phase (aqueous) was removed by aspiration and partially evaporated under a gentle stream of gaseous N\(_2\) to remove most of the MeOH. The sample was then lyophilized to dryness. The organic phase containing lipids (lower phase) was isolated and evaporated to dryness by blowing a gentle stream of N\(_2\) over the sample. This sample was then weighed to obtain total lipid content.

2.5. Preparation of samples for NMR

Dried extracts recovered from the aqueous fraction were reconstituted in 10 ml of H\(_2\)O and treated with Chelex 100 to remove divalent ions. The Chelex 100 was removed by centrifugation and the sample was lyophilized a second time. This dried extract was then reconstituted in H\(_2\)O:\(^{2}\)H\(_2\)O (5:1 v/v) with 10 mM EDTA (pH 8.0).

Lipid extract samples were prepared using a three-part solvent system containing CDCl\(_3\), MeOH, and aqueous Cs\(_2\) (EDTA) as described elsewhere [13,14]. Samples were placed in 10 mm NMR tubes with a capillary tube containing an external standard for quantitation purposes. Methylene diphosphonic acid (MDPA) or a solution of inorganic phosphate was used as a standard for \(^{31}\)P aqueous or lipid samples, respectively, while MeOH was used for \(^{13}\)C NMR.

Fig. 1. Schematic diagram for the kinetic model representation depicting the biosynthesis of PtdC and PtdE from \(^{13}\)C-labeled choline and ethanolamine (asterisks denote label positions). Together, reactions A and B are the CDP-choline pathway (de novo) for synthesis of PtdC. A represents the uptake of choline from blood and its phosphorylation by choline kinase in liver. B represents the combined action of CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase. Reactions C and D are the CDP-ethanolamine pathway (de novo) for PtdE synthesis. C represents the uptake of ethanolamine from the blood and its phosphorylation by ethanolamine kinase in liver. D represents the combined action of CTP:phosphoethanolamine cytidylyltransferase and CDP-ethanolamine:1,2-diacylglycerol ethanaminephosphotransferase. Reaction E represents the PEMT pathway which results in C2-labeled PtdC. Reactions B', D', and E' represent the fluxes between the fast and slow metabolite pools as described in the text.
2.6. NMR experiments

High-resolution proton-decoupled $^{31}$P and $^{13}$C NMR spectra of liver extracts were acquired in field-lock mode using a 10 mm broadband probe operating at 145.8 and 90.6 MHz, respectively. For lipid extracts, the sample temperature was regulated at 293 K to minimize evaporation and to optimize spectral resolution for $^{31}$P NMR as demonstrated previously [15]. $^{31}$P NMR of lipid samples were acquired under full $T_1$ relaxation conditions without nuclear Overhauser enhancement (NOE) using a gated $^1$H-decoupling sequence. Data acquisition parameters included a 90° pulse, 874 Hz spectral bandwidth, 2.3 s acquisition time, 15.3 s interpulse delay, and approx. 3 h of signal averaging. $^{13}$C NMR of lipid samples were acquired with NOE using a 60° pulse, 20 kHz spectral bandwidth, 1.6 s acquisition time, 4.6 s interpulse delay, and approx. 14 h of signal averaging. The aqueous samples were regulated at 298 K and acquired using a gated $^1$H-decoupling sequence with NOE for both $^{13}$C and $^{31}$P. $^{31}$P data were acquired using a 50° pulse, 8772 Hz bandwidth, 1.9 s acquisition time, 8.0 s interpulse delay, and approx. 4 h of signal averaging. C-13 aqueous data were acquired using a 60° pulse, 20 kHz bandwidth, 1.6 s acquisition time, 4.6 s interpulse delay, and approx. 17 h of signal averaging. NMR data were processed using a Macintosh PowerPC and MacFid software (Tecmag, Houston, TX, USA). $^{31}$P data for lipid and aqueous samples were processed using 8K or 64K total points, respectively, and Fourier transformed without apodization of the free induction decay (FID) signal. All $^{13}$C data were processed using 128K total points, exponential multiplication yielding 1 Hz line broadening, and Fourier transformation.

For quantitative purposes, the $^{31}$P and $^{13}$C NMR signal intensities of the aqueous metabolites Pch, Pth, glycerophosphocholine (GPC), and glycerophosphoethanolamine (GPE) were corrected for $T_1$ saturation and NOE. This procedure was not necessary for $^{31}$P spectra of lipid extracts since these spectra were acquired under fully relaxed conditions ($\geq 5T_1$) and without NOE. For $^{13}$C NMR of lipid samples we found that any differences in $T_1$ saturation and NOE between the C1, C2, and methyl carbons of PtdC, or the C1 and C2 carbons of PtdE, were insignificant under the parameters used to acquire the data. This was determined from the measurement of signal intensity ratios for the head group carbons of PtdC and PtdE in liver samples obtained without infusion of labeled substrates (0 min time point). In these samples the intensity ratios were 1:1:3 for PtdC (C1:C2:methyl) and 1:1 for PtdE (C1:C2) which are the ratios expected for natural abundance intensities. Thus our experimental acquisition conditions do not perturb the signal intensity ratios. In separate experiments, the $^{31}$P NMR signal intensities for the external references (MDPA and P) were calibrated against standard solutions of known concentrations of PtdC (for lipid samples) or Pcho (for aqueous samples). The signal intensities from the external references could then be used to quantitate metabolites from the $^{31}$P NMR signals in experimental samples.

2.7. Data analyses

Measurements of the $^{31}$P NMR spectral intensities from aqueous and lipid extracts yielded metabolite concentrations, while $^{13}$C NMR spectral intensities provided an assessment of the $^{13}$C enrichment of the metabolite pools. The $^{13}$C enrichment factor (EF) is defined as the factor by which the $^{13}$C concentration is increased above the natural abundance level of 1.1% (i.e. EF × 0.011 = $^{13}$C fractional enrichment). The methyl-choline carbons of PtdC and the C1 carbon on the PtdE head group (−O-CH$_2$−) are present at natural abundance concentrations. NMR signals from these carbons were used as references to measure the $^{13}$C enrichments at the C1 (−O-CH$_2$−) and C2 (−CH$_2$-N) carbons of the PtdC head group, and the C2 carbon (−CH$_2$-N) of the PtdE head group. For aqueous samples, the fairly low concentrations of Pcho and Peth (∼1 μmol/g liver) prohibited detection of the natural abundance $^{13}$C signals from these molecules; only $^{13}$C enriched carbon sites were detected. This was confirmed in spectra obtained from rats after infusion with unlabeled substrates. Hence, an external sample of 5% MeOH was calibrated against standard samples containing known concentrations of Pcho and Peth. Then in the experimental samples the signal intensity of Pcho or Peth relative to this MeOH standard provided a measure of the ‘apparent’ concentration (the
intensity is a function of both concentration and $^{13}$C enrichment). The EF was then determined from the ratio of apparent concentration (derived by $^{13}$C NMR) to the true concentration as measured by $^{31}$P NMR in these same samples.

Data analyses were performed using the appropriate Student’s $t$-test or ANOVA, and all data were considered statistically significant at the 95% confidence level ($P \leq 0.05$). Fits of kinetic models to experimental data employed a non-linear least-squared regression algorithm. The goodness of the fit for different models was evaluated by comparing the chi-square statistic.

2.8. Kinetic models

The kinetic models were coded on a PC using a numerical integration package – Advanced Computing Simulation Language (ACSL level 11, MGA Associates, Concord, MA, USA). The analyses were conducted using the $^{13}$C EF data, but identical results would be obtained if the analyses used absolute concentrations of metabolites. A schematic diagram of the modeled reactions is shown in Fig. 1. The choline and ethanolamine pools in the figure represent blood pools, and are assumed to be 100% $^{13}$C labeled at the appropriate carbon positions and inexhaustible. These substrates are assumed to be transported into liver and phosphorylated to yield Pcho and Peth. In this model, the only source of substrates for generating new Pcho and Peth are 100% $^{13}$C-labeled choline and ethanolamine from the plasma. The separation of the pools of Pcho, Peth, and PtdE into ‘fast’ and ‘slow’ compartments was necessary to accurately simulate experimental data, as described in Section 3. For the simplest model each reaction was assumed to progress at a constant rate expressed in $\mu$mol/h/g of liver. The model allows flux from the fast to the slow compartments, but not from the slow to the fast compartments. We also considered several alternative versions of this model which will be addressed in Section 4. In one particular version, for which the results are presented in various figures, we allowed the reaction rates to increase linearly during the experimental time course.

3. Results

Fig. 2 shows portions of the $^{13}$C NMR spectra from rat liver lipid extracts before and 60 min after
infusion with $^{13}$C-labeled substrates. These data demonstrate that the incorporation of $^{13}$C label into the phospholipid pools is detectable in our experimental protocol. The C1, C2, and N(CH$_3$)$_3$ carbons of the choline head group of PtdC resonate at 59.4, 66.7 and 54.4 ppm, respectively. These are clearly discernable from the C1 and C2 carbons of the PtdE head group which resonate at 62.2 and 40.9 ppm, respectively. In the spectrum obtained prior to infusion (Fig. 2B), the expected natural abundance signal intensity ratios are observed for the head group carbons of PtdC and PtdE (1:1:3 for C1:C2:N(CH$_3$)$_3$ of PtdC and 1:1 for C1:C2 of PtdE). After $^{13}$C label infusion (Fig. 2A), the intensity ratio for the PtdC head group signals is 2.6:1.6:3.0, and that for the PtdE head group signals is 1.0:9.4.

### 3.1. Concentrations of liver metabolites

The concentrations of liver lipid and aqueous metabolites are displayed in Table 1. [PtdC] and [PtdE] were measured from the $^{31}$P NMR spectra of the lipid extracts. A detailed description of this analysis and a representative spectrum were given in previous publications [13,15]. The liver concentrations of Pcho, Peth, GPC, and GPE were measured from the $^{31}$P NMR spectra of aqueous extracts; a representative spectrum depicting these signals is shown in Fig. 3. The pools of Pcho, Peth, PtdC, and PtdE remained constant from 0 to 60 min during the infusion of substrates (ANOVA, $P < 0.05$). The liver concentrations for each of these metabolites averaged over all time points were: [Pcho] = 0.9 ±

### Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pcho</td>
<td>0.81 ± 0.21</td>
<td>1.00 ± 0.26</td>
<td>0.91 ± 0.30</td>
<td>0.81 ± 0.30</td>
<td>0.87 ± 0.16</td>
</tr>
<tr>
<td>Peth</td>
<td>1.12 ± 0.61</td>
<td>0.92 ± 0.04</td>
<td>1.12 ± 0.25</td>
<td>0.90 ± 0.37</td>
<td>1.08 ± 0.20</td>
</tr>
<tr>
<td>PtdC</td>
<td>29.46 ± 6.75</td>
<td>24.34 ± 2.26</td>
<td>24.35 ± 2.68</td>
<td>23.94 ± 2.76</td>
<td>25.81 ± 5.42</td>
</tr>
<tr>
<td>PtdE</td>
<td>11.22 ± 3.98</td>
<td>10.31 ± 0.98</td>
<td>10.34 ± 1.80</td>
<td>10.34 ± 1.24</td>
<td>11.43 ± 2.11</td>
</tr>
<tr>
<td>GPC</td>
<td>0.47 ± 0.20</td>
<td>0.44 ± 0.12</td>
<td>0.61 ± 0.16</td>
<td>0.54 ± 0.23</td>
<td>1.16 ± 0.58$^*$</td>
</tr>
<tr>
<td>GPE</td>
<td>0.35 ± 0.09</td>
<td>0.31 ± 0.06</td>
<td>0.34 ± 0.06</td>
<td>0.26 ± 0.12</td>
<td>0.49 ± 0.04$^*$</td>
</tr>
</tbody>
</table>

Asterisks denote a significant difference in a metabolite concentration with regard to time (ANOVA; $P < 0.05$); these data were determined to be significantly different from other time points by Fisher’s post-hoc analysis.

$^*$Values are μmol/g liver (mean ± S.D.; $n = 4$–5) measured by $^{31}$P NMR from 0 to 60 min during infusion with choline and ethanolamine.
0.2; [Peth] = 1.0 ± 0.3; [PtdC] = 25.5 ± 4.3; [PtdE] = 10.7 ± 2.1 μmol/g liver (mean ± S.D.; n = 22 or 23 for aqueous or lipid metabolites, respectively). n-Values differ due to the loss of one of the aqueous samples. The concentrations of GPC and GPE showed significant differences during the experimental time course as determined by ANOVA (P < 0.05). A Fisher’s post-hoc analysis indicated that the [GPC] and [GPE] were significantly increased at 60 min versus all other time points.

The 13C spectra of aqueous extracts obtained before and 60 min after infusion with 13C-labeled substrates are shown in Fig. 4. Natural abundance signals from Pcho and Peth are not observable (before infusion, Fig. 4B). Label incorporation into these metabolites, however, is detectable after infusion with 13C enriched substrates (Fig. 4A). Additionally, 13C incorporation into the carbonyl carbon of betaine was also observed (169.9 ppm; not shown in Fig. 4). The betaine signal intensities (mean ± S.E.) normalized per gram of liver were plotted versus the experimental time course as shown in Fig. 5. The 0 min time point was assigned a signal intensity value of zero which reflects the fact that the betaine signal was below detection limits in the spectra obtained prior to infusion of 13C-labeled substrates (i.e. natural abundance 13C betaine was not observable).
The change in this signal intensity indicates a linear increase in betaine concentration from 0 to 60 min during substrate infusion ($r^2 = 0.97$). GPC and GPE were below levels of detection by $^{13}$C NMR.

3.2. $^{13}$C enrichment of metabolite pools and kinetic analyses

Figs. 6 and 7 show the $^{13}$C EFs for Pcho, Peth, PtdC, and PtdE during infusion of labeled substrates. The metabolites receiving label from choline are shown in Fig. 6, while those receiving label from ethanolamine are shown in Fig. 7. Data points are from the NMR experimental measurements (mean ± S.E.), while the lines represent the kinetic model predictions. Separate analyses were performed assuming one of two possible conditions: (i) the reaction rates were held constant, or (ii) the reaction rates were initially set at zero and allowed to increase linearly over the 60 min time course. Model predictions for both assumptions are displayed in Figs. 6 and 7 and identified as 'cf' (constant flux) or 'uf' (upregulated flux). The reaction fluxes used to create the simulation curves are shown in Table 2. The letters in this table refer to the reaction pathways identified in Fig. 1. Interestingly, the kinetic model required two pools for Pcho, Peth, and PtdE in order to obtain a reasonable fit of the experimental data (this is further explained in Section 4). In our first approximation (Model-cf), the flux was unidirectional from the fast to the slow compartments. Thus the size of the slow compartments may increase during the 60 min infusion of substrates. The changes in compartment size as predicted by the models, and the initial ($t = 0$) proportion of the metabolites in the fast pools are also shown in Table 2. The model-predicted changes in the concentrations of Pcho, Peth, and PtdE were small. The largest change was for Pcho, whereby the increase in pool size was calculated to be about 11%. For Model-uf we allowed a...
two-way exchange between the fast and slow compartments for Peth in order to provide a better fit of the experimental data. Thus the pool size for Peth in this model is constant. The small changes in the model-predicted pool sizes are not detectable with the NMR method for estimating metabolite concentrations. Thus, as mentioned previously, the concentrations of all metabolites as measured by NMR (Table 1) were constant over the 60 min time course within experimental error.

All metabolites show a steady increase in $^{13}$C label incorporation over the 60 min infusion time course, with Pcho attaining the highest enrichment (Figs. 6 and 7). The EF for Pcho at 60 min is significantly greater than for Peth (17.5 ± 2.1 vs. 3.6 ± 0.5; mean ± S.E.). Model-cf does not predict well the data for these metabolites, which show an initial lag in labeling followed by much greater labeling at later time points. This prompted us to re-examine the model assuming that certain fluxes were upregulated in the reaction pathways (Model-uf). This latter model predicts the above-mentioned features of the experimental data. Other alternative models that also predict a lag followed by increased labeling for these metabolites are explained in Section 4.

For the ethanolamine-containing metabolites, the EFs are significantly greater for PtdE than for its precursor, Peth, at all time points ($P < 0.03$; Fig. 7). At 60 min, the EF for C2-PtdE is 2 times the value for C2-Peth (7.7 ± 0.8 vs. 3.6 ± 0.5). The data for C2-PtdE are also fairly linear, as are the data for C2-PtdC. Thus upregulating the flux in these pathways (Fluxes D and E) was not necessary, and both models (cf and uf) provide a good fit to the data.

The data for C1-PtdC and C2-PtdC are shown together in Fig. 8 along with the prediction lines

<table>
<thead>
<tr>
<th>Flux</th>
<th>Model-cf (constant flux)</th>
<th>Model-uf (upregulated flux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A$^{b,c}$</td>
<td>0.31</td>
<td>0 → 1.05</td>
</tr>
<tr>
<td>B</td>
<td>0.21</td>
<td>0 → 0.70</td>
</tr>
<tr>
<td>C$^{d}$</td>
<td>0.10</td>
<td>0 → 0.35</td>
</tr>
<tr>
<td>Cs</td>
<td>0.94</td>
<td>1.04</td>
</tr>
<tr>
<td>D</td>
<td>0.92</td>
<td>1.04</td>
</tr>
<tr>
<td>D$^{d}$</td>
<td>0.02</td>
<td>0 → 0.06</td>
</tr>
<tr>
<td>E</td>
<td>0.12</td>
<td>0.14</td>
</tr>
<tr>
<td>E$^{d}$</td>
<td>0.80</td>
<td>0.90</td>
</tr>
<tr>
<td>Change in slow pool size for Pcho (µmol/g liver)$^{e}$</td>
<td>0.88 → 0.98</td>
<td>0.88 → 0.94</td>
</tr>
<tr>
<td>Initial percent Pcho in fast compartment$^{f}$</td>
<td>1.5%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Change in slow pool size for Peth (µmol/g liver)</td>
<td>1.03 → 1.05</td>
<td>1.03 (two-way exch)</td>
</tr>
<tr>
<td>Initial percent Peth in fast compartment$^{g}$</td>
<td>0.01%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Change in slow pool size for PtdE (µmol/g liver)</td>
<td>10.73 → 11.53</td>
<td>10.73 → 11.27</td>
</tr>
<tr>
<td>Initial percent PtdE in fast compartment$^{d}$</td>
<td>1.2%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Ratio of PtdC derived from de novo vs. PEMT pathways</td>
<td>64:36</td>
<td>71:29$^{e}$</td>
</tr>
<tr>
<td>Chi-square</td>
<td>16.1</td>
<td>5.4</td>
</tr>
</tbody>
</table>

$^{a}$Models are described in Section 2. The number of fluxes fit by the model is 5; other fluxes were then derived by difference (i.e. Flux B = A - B'). For Model-cf, the number of independent parameters was 8. For Model-uf, since each independent upregulated flux (A, B', and D') needs values for both the initial flux and the rate of increase, the number of independent parameters was 11. Note, however, that the best fit to the experimental data was achieved by setting the initial flux for each upregulated flux to zero. The simulation curves are shown in Figs. 6-8 along with the experimental data.

$^{b}$Reaction fluxes are given in µmol/h/g liver. Letters refer to the metabolic pathways depicted in Fig. 1.

$^{c}$Model-uf assumed upregulated fluxes and allowed a two-way exchange for Peth between the fast and slow pools. For all other cases the models allowed for flux from the fast compartment to the slow compartment only, so the concentration of the slow compartment increased during the 60 min infusion of substrates. The model-calculated changes in these metabolite concentrations are shown along with the calculated percentages of these metabolites in the fast compartments (relative to the total pool size).

$^{d}$Identifies independent parameters.

$^{e}$For Model-uf, the percent of PtdC derived by each pathway was calculated using Flux B = 0.35. This is the 'average' value calculated from the range given for Flux B.
3.3. Metabolic pathway analyses of PtdC biosynthesis

The most interesting reaction pathways are denoted as B and E in Fig. 1 and involve the fluxes into PtdC from Pchol (de novo pathway) and from PtdE (PEMT pathway). The total flux into PtdC can be represented by \( B+E \), and the fraction of PtdC derived from choline is simply \( B/(B+E) \). For Model-cf, the total flux into PtdC is predicted to be 0.33 \( \mu \text{mol/h/g liver} \) and the percentage ratio of the de novo pathway flux to PEMT flux is predicted as 64:36 (Table 2). For Model-uf, the fluxes are variable over the time course with the initial flux set at zero. If one assumes an average flux over the 60 min time course of 0.35 \( \mu \text{mol/h/g} \) for Flux B (midpoint in the range), then the fraction of PtdC derived via the de novo pathway is 0.71 (71%).

The concentrations of newly synthesized phospholipids with specific \(^{13}\text{C}\) labels can be calculated from the \(^{13}\text{C}\)-measured EFs and the \(^{31}\text{P}\)-measured metabolite concentrations according to Eq. 1.

\[
[Cn-PL]_{\text{new}} = (0.011)[PL](EF_{cn}-1)
\]

Here \( Cn-PL \) is the phospholipid labeled at the \( n \)th carbon (i.e. C1-PtdC), \( EF_{cn} \) is the enrichment factor measured for this specific carbon, and \( [PL] \) is the total concentration of this particular phospholipid pool. The concentrations of newly synthesized PtdE and PtdC at various time points following infusion with labeled substrates are given in Table 3. The C1-PtdC label provides a measure of the PtdC derived from the de novo pathway while the C2-PtdC provides a measure of the PEMT pathway contribution. The total amount of newly synthesized PtdE was calculated by summing the pools of C2-PtdE and C2-PtdC, since the C2-PtdC was derived from the labeled PtdE pool. These data show that the percentage of PtdE that is converted to PtdC via the PEMT pathway is constant at about 13% from 30 to 60 min. The total amount of newly synthesized PtdC can be calculated using Eq. 1. It follows from Eq. 1 that the fraction of PtdC derived from each pathway is simply calculated as:

\[
(F_{E1}-1)/(EF_{c1} + EF_{c2}-2)
\]
where $i$ represents C1 for the de novo pathway or C2 for the PEMT pathway. Table 3 shows the percentages of PtdC derived from these pathways at each time point. The proportion of PtdC derived from either pathway is stable after 45 min and is about 70:30 (de novo vs. PEMT). This compares favorably with the model predictions.

### 4. Discussion

An experimental design employing a specific isotopic labeling scheme in conjunction with NMR spectroscopy has enabled a kinetic analysis of phospholipid biosynthesis in rat liver. The metabolic pathways involved in the biosynthesis of PtdC and PtdE from choline and ethanolamine were discerned simultaneously in a single experiment. The NMR methodology provides superior specificity to radioisotope methods but suffers from inherently lower sensitivity. Thus the substrates, choline and ethanolamine, were administered in much larger quantities than in previous radioisotope tracer studies. The liver concentrations of CDP-choline and CDP-ethanolamine were below NMR detection limits (for both 31P and 13C) and thus 13C labeling of these metabolites was not observed. Pool sizes for CDP-choline and CDP-ethanolamine in rat liver were previously reported to be approx. 0.011 and 0.030 μmol/g liver, respectively [1,2] (calculated from these literature reports assuming an 8 g liver). An advantage of the NMR technique, however, is its capability for metabolic pathway analyses. In a very straightforward manner, our data revealed that about 70% of PtdC was derived through the de novo (CDP-choline) pathway while 30% was through the PEMT pathway. This compares favorably with published estimates for the activity of these pathways as 60–80% de novo and 20–40% PEMT [4,16–19].

#### 4.1. Channeling

Most interestingly, our data suggest that there is ‘channeling’ of metabolites through the biosynthetic pathways, or compartmentation of specific processes. In other words, metabolites derived from exogenous choline and ethanolamine do not thoroughly mix with the intracellular pools, but they are preferentially utilized in the pathway for phospholipid biosynthesis. Our data suggest that the CDP-choline pathway, the CDP-ethanolamine pathway, and the PEMT pathway are all channeled metabolic processes.

In order to obtain a reasonably good fit of the experimental data ($^{13}$C EFs, Figs. 6 and 7), it was necessary for the kinetic model to include two pools for $\text{Pcho}$, $\text{Peth}$, and $\text{PtdE}$ (Fig. 1). Without the inclusion of these fast/slow pools, the model fit to the data was extremely poor. The ‘fast’ pool receives label from the precursor, and is used when making the next metabolite in the pathway. The ‘slow’ pool can only receive label from the fast pool. This scheme is necessary, otherwise the precursor needs to be more $^{13}$C enriched than a single pool allows in order to attain sufficient labeling in the product metabolite.

The necessity for two metabolite pools is most readily illustrated for $\text{Peth}$. The $[\text{PtdE}]$ is approx.
10 times greater than the [Peth], yet the PtdE pool attains greater $^{13}$C enrichment than its precursor Peth (EF = 7.7 vs. 3.5 at 60 min). This indicates that ethanolamine is 'channeled' into the phospholipid pool, and newly synthesized Peth is preferentially used for PtdE synthesis. Our data suggest that channeling also occurs in the CDP-choline pathway. For example, when Model-cf using a single pool of Pcho was fit to the C1-Pcho data, the resulting EF of C1-PtdC yielded only 1.03, instead of the experimental value of 2.2 (results not shown). Thus the kinetic model required a fast and slow pool for Pcho suggesting that newly synthesized Pcho is preferentially used for PtdC biosynthesis. One caveat in this interpretation is that our analyses cannot account for base-exchange reactions between PtdC or PtdE with other existing glycerophosphatides, such as phosphatidylserine. This could provide an additional pathway to generate labeled PtdC or PtdE whereby formation of the corresponding precursor (Pcho or Peth) is bypassed. Consequently, this would lead to an overestimation of the de novo pathway activity. This is an unlikely explanation of our data, however, since the contribution of the base-exchange pathway is considered to be minor [20]. Previous studies have shown that base-exchange between phosphatidylserine and PtdE in liver is negligible in comparison with the CDP-ethanolamine pathway [2]. Although little is known about these base-exchange pathways, phosphatidylserine synthases I and II operate by base-exchange mechanisms primarily in the direction of phosphatidylserine biosynthesis [20–22]. Thus this would not introduce additional label into the pools of PtdC or PtdE. Such a mechanism, however, could affect the $^{13}$C enrichment of the intracellular pool(s) of base (choline or ethanolamine) in unpredictable ways. For instance, biosynthesis of phosphatidylserine via phosphatidylserine synthase II involves the exchange of serine for the ethanolamine base in PtdE. The released ethanolamine molecule would affect the isotopic enrichment of the intracellular ethanolamine pool in opposite ways dependent upon whether a labeled or unlabeled molecule of PtdE was used in the reaction mechanism. Also unclear is the extent to which this ethanolamine molecule is recycled back into the phospholipid pool. Our model analyses do not enable an assessment of these base-exchange mechanisms.

Others have also provided evidence for channeling of intermediates into phospholipid biosynthetic pathways from radioisotope studies in glioma cells [8,9], heart [10], and liver [1,2,11]. In 1972–73, Sundler et al. [1,2] conducted studies of phospholipid biosynthesis in rat liver using radiolabeled choline and ethanolamine and concluded that the data did not fit a simple precursor-product relationship. They suggested that exogenous choline and ethanolamine were metabolized differently from the endogenous metabolite pools. A more recent study by Bladegroen et al. [9] used permeabilized cells and different radiolabeled substrates to examine whether channeling occurs in various cell types. They concluded that channeling is involved in phospholipid synthesis in glioma cells and fibroblasts, but their results for hepatocytes were inconclusive since these cells were not amenable to study by their methods of permeabilization. Our isotopic NMR analyses corroborate the early work of Sundler et al. and demonstrate that liver PtdC and PtdE synthesis are channeled processes in rat liver in vivo. Such channeling is not a concentration dependent phenomenon since our studies were conducted at much higher concentrations than the radioisotope tracer studies.

The channeling mechanism presumably involves cooperation among various enzymes and/or compartmentation of several pathway components. This view is consistent with the cellular compartmentation reported for the various enzymes involved in phospholipid biosynthesis, but the entire mechanism is complicated and must involve transport processes. Choline and ethanolamine enter the cell at the plasma membrane and are phosphorylated by a protein kinase in the cytosol. Choline and ethanolamine kinase activity in mammalian cells is known to reside on the same protein that is primarily cytosolic, but there are also reports of various protein isoforms with a high substrate specificity [23]. The latter enzymes in the pathway may provide a channeled mechanism through compartmentation. Most noteworthy are CTP:phosphocholine cytidylyltransferase (CCT) and CTP:phosphoethanolamine cytidylyltransferase (ECT), the rate-controlling enzymes for the CDP-choline and CDP-ethanolamine pathways, respectively. CCT exists in soluble form in the cytosol but is activated by lipids which may occur through its association with membranes in vivo.
Recent studies show that mammalian cells have stores of CCT in the nucleus that may be translocated to the endoplasmic reticulum when PtdC biosynthesis is stimulated [25–28]. Nuclear localization may vary among cell types. In CHO cells, CCT is concentrated in the nucleus but studies in rat hepatocytes show that CCT is primarily distributed in the cytosol with lesser amounts in the nucleus [25]. Although less is known about ECT, its distribution in hepatocytes is also not uniform. Studies have shown that ETC is absent from the nucleus but is present in the cytosol and the rough endoplasmic reticulum [29,30]. Thus its activity may also involve reversible association with lipids of the endoplasmic reticulum which could bring it in close proximity to ethanolaminephosphotransferase, the final enzyme in the CDP-ethanolamine pathway. Both ethanolamine-phosphotransferase and cholinephosphotransferase are integral membrane proteins of the microsomal cell fraction, and the bulk of activity is thought to reside in the endoplasmic reticulum [31,32]. Thus co-localization of some specific enzymes is consistent with channeling of the metabolic pathways.

We show for the first time that channeling may also be involved in the PEMT pathway for interconversion of PtdE into PtdC. The fast/slow pool analysis was necessary for PtdE, as it was for Pcho and Peth. When the model analysis was conducted with only a single pool of PtdE, the predicted line did not fit the data (Fig. 8). Thus the entire pool of PtdE is not available to the PEMT pathway, but rather, the newly synthesized PtdE derived by the CDP-ethanolamine pathway is the preferred substrate. This result may be simply interpreted in terms of availability of substrate, whereby preexisting membrane-bound PtdE is not as accessible to the PEMT enzyme as is the newly synthesized PtdE. Alternatively, it may suggest that PtdE biosynthesis is somehow linked to the PEMT pathway through compartmentation or cooperativity between enzymes. With regard to compartmentation, the ECT enzyme is distributed between the cytosol and the rough endoplasmic reticulum, and reversible membrane binding may be involved in its regulation [20,30]. PEMT exists as two isoforms in liver which are encoded on the same gene; PEMT1 activity resides with the endoplasmic reticulum and PEMT2 is localized to the mitochondrial-associated membrane fraction [33]. Thus it seems likely that our experiment is showing the activity of the CDP-ethanolamine pathway and PEMT1, which are co-localized in the endoplasmic reticulum and appear to be functioning in concert to produce both PtdE and PtdC. Such findings may have important implications with regard to cell functions. Indeed, recent studies have shown that the PtdC species derived by the CDP-choline and PEMT pathways are unique due to differences in the composition of the acyl groups [34]. These different molecular species may play different roles and, thus, the proportions of PtdC derived from these two pathways may have functional significance. Studies by Vance and coworkers have demonstrated a reciprocal relationship between the activity of PEMT2 and the CDP-choline pathway. Furthermore, they suggest that this may play a regulatory role in cell proliferation [35]. Expression of PEMT2 in a hepatoma cell line resulted in downregulation of the CDP-choline pathway and suppression of cell growth [5]. Studies of perinatal rat liver [36] and a model of regenerating liver [37] also showed an inverse correlation between PEMT2 expression and hepatocyte proliferation. Thus our finding that the PEMT pathway is channeled may have important implications with regard to cell functions, particularly cell growth processes.

4.2. Analysis of Pcho and Peth data

By the criteria that a fit should produce a chi-square value approximately equal to or less than the number of degrees of freedom (d.f.), both Model-cf (d.f. = 17) and Model-uf (d.f. = 14) produce acceptable results. However, a prominent feature of the data, namely the non-linearity and the distinct ‘lag’ in the labeling of Pcho, Peth, and C1-PtdC, was not fit well by Model-cf. We checked to ensure that our Pcho data were not erroneously evaluated due to overlapping NMR resonances from GPC, especially since the [GPC] was found to be significantly elevated at the 60 min time point. A standard solution of Pcho plus GPC was prepared and analyzed by 13C NMR. Data showed that the C1-carbon of Pcho is well resolved from the C1-choline signal of GPC (Δδ=1.4 ppm; Pcho is at lower frequency). Thus the lag in the labeling of Pcho and Peth is real. Therefore, we considered several alternative models.
which might account for these data: (i) the $^{13}$C enrichment of the substrate blood pools is not at steady state but gradually increases during the experiment; (ii) the $^{13}$C labels entering from the blood are first diluted into the intracellular pools of choline and ethanolamine before being used as substrates for biosynthesis; (iii) the reaction fluxes are not constant but are upregulated during the experimental time course. All of these scenarios were explored and do provide a better fit of the model to the data; however, the idea of upregulated fluxes seems most appropriate based upon information from the literature. Thus we chose to display those model results herein. As a first approximation, Model-cf assumes that our infusion of choline and ethanolamine produces a rapid labeling of the blood and intracellular pools to nearly 100% $^{13}$C. This assumption seems reasonable since the rat plasma choline concentration is about 10 μM and mammals have a high capacity for choline clearance from the blood [38-40]. The ethanolamine level in rat serum is about 30 μM [41], and intracellular pool sizes of choline and ethanolamine are quite low (approx. 0.2 μmol/g liver) relative to our infusion amounts (0.5 μmol/min) [1,2]. For these reasons, alternative model (i) mentioned above was considered to be an unlikely explanation for the lag in labeling of Pcho and Peth. The second alternative interpretation mentioned above is not amenable to analysis by our methods due to the low levels of intracellular choline and ethanolamine. This particular interpretation, however, would be contradictory to previous reports which suggest that exogenous choline and ethanolamine do not mix with the endogenous pools [1,2]. Also, there are various reports providing evidence for pathway channeling [8-11]. In our simple model (Model-cf) choline and ethanolamine are assumed to enter the cell and be directly phosphorylated (a channeled process). Although we do not have direct evidence for channeling at this particular pathway step, channeling of metabolites at this early part of the pathway would be consistent with our evidence for channeling at later steps and with other literature reports. The last alternative assumes that certain reaction fluxes are linearly upregulated and the results shown in Figs. 6-8 are quite good. Of course, it is possible that all three of these scenarios are operative and the model can be made to fit the data with contributions from each. A further discussion of these results with regard to differences observed between the CDP-choline and CDP-ethanolamine pathways is given in Section 4.4.

One might speculate that upregulated fluxes could occur in response to increased enzyme activity or an increase in substrate availability. With regard to the latter, others have shown that the rate of PtdE synthesis is dependent upon the extracellular ethanolamine concentration in COS-7 cells [42] and isolated rat hepatocytes [41]. Since we did not measure plasma choline or ethanolamine we cannot comment on this possible mechanism; however, the high capacity for uptake of these substrates in mammals may rapidly stabilize the blood levels. For example, cats infused with choline at rates ranging from 0.8 to 5 μmol/min/kg showed an increase in the plasma choline concentration but a new steady state level was reached with a half-time of about 7 min or less [39]. Based upon this report it seems likely that the blood choline concentration increases during the time frame prior to acquiring our first data point, but it should be at steady state at all subsequent data collection times. Also note that the kinetic model predictions (Table 2) show increases in the intracellular pools for intermediary pathway metabolites, although these changes in concentration were small and within the experimental error of the NMR measurements. Thus an increase in the extracellular substrate concentrations and the intracellular metabolite pools is one possible mechanism for upregulation of pathway fluxes, but changes in enzyme activities that are independent of substrate concentrations may also play a role (i.e. allosteric effects, covalent modifications).

4.3. Phospholipid turnover (GPC and GPE data)

Our kinetic model analysis assumes that any contribution to the CDP-choline or CDP-ethanolamine pathways from turnover of the phospholipid pools is negligible. Recycling of metabolites would certainly compromise the analysis, particularly if these metabolites are $^{13}$C-labeled. With regard to this latter issue, our assumption seems warranted since the label enrichment of the PtdC pool is approx. 2% and thus only about 2% of compounds produced from PtdC are expected to contain label. If GPC is assumed to
be the major degradation product, then this metabolite would attain a 2% labeling at most, based upon a purely statistical argument. Recycling of this metabolite pool back into phospholipids could provide another input to the CDP-choline pathway; however, metabolite channeling might also prevent such a possibility. Regardless, the contribution of $^{13}$C to the Pcho pool from turnover of the phospholipid pool is likely to be small compared to the input flux from the infused exogenous substrates.

Additionally, GPC and GPE were not observed by $^{13}$C NMR. The levels of these metabolite pools are below detection by natural abundance $^{13}$C NMR, but significant $^{13}$C enrichment might make them NMR visible. Based upon the pool size and the fact that GPC was not observed by $^{13}$C NMR, we estimate that GPC must have a $^{13}$C enrichment of less than 1.25 (1.38% labeled). This again provides further support for our model assumption which neglects the recycling of phospholipid degradation products which contain any significant $^{13}$C enrichment. This does not preclude the existence of phospholipid turnover during our experiment, but rather, the contribution of $^{13}$C label to the metabolite pools from such turnover is presumed insignificant. On the other hand, the recycling of metabolites at natural abundance $^{13}$C levels (referred to as unlabeled metabolites below) might dilute the $^{13}$C enrichment of the Pcho and Peth pools. If this happened to a significant degree, and if these degradation products were available to the biosynthetic pathways (i.e. if channeling does not prevent their entry into the pathway), then the model would underestimate the total pathway fluxes. The extent of such recycling is difficult to assess and was neglected in our model, but even if unlabeled recycling were significant, the pathway fluxes reported herein would still represent the rate of synthesis of phospholipids from extracellular substrates. Also, it is important to note that the recycling of unlabeled molecules would not compromise our interpretation of the data with regard to pathway channeling.

Others have observed increases in GPC and GPE following upregulation of PtdC synthesis by addition of excess phospholipid in HeLa cells [43] and over-expression of CCT in COS cells [44]. They attributed this to an increase in turnover as a mechanism to maintain phospholipid content. We measured the concentrations of liver GPC and GPE since these metabolites may be indicative of phospholipid turnover. Our data show that these metabolites are not significantly increased until 60 min following substrate infusion. Early reports of phospholipid turnover in rat liver using radioisotope methods indicate a half-life of 6-10 h ([45] and references therein).

It is interesting to compare the amounts of newly synthesized phospholipids and the increases observed in GPC and GPE. At 60 min, the total amount of newly synthesized PtdC was approx. 0.5 μmol/g liver (Table 3) and GPC was increased by about 0.7 μmol/g liver, relative to the 0 min time point (Table 1). The newly synthesized PtdE amounted to about 1 μmol/g liver, but there was a much smaller increase in GPE of approx. 0.2 μmol/g liver. These values may indicate that the processes of synthesis and degradation are more closely matched for PtdC than for PtdE. Perhaps other degradation pathways are more important for PtdE but the current data do not enable this evaluation. Within the experimental error of the NMR measurement, the total pool sizes for PtdC and PtdE did not change during the time course of the study (Table 1).

4.4. Rates of PtdC and PtdE synthesis

The de novo pathways for biosynthesis of PtdC and PtdE display uniquely different kinetics. The $^{13}$C enrichment of Pcho and C1-PtdC show similar non-linear trends (Figs. 6 and 8). The Peth and C2-PtdE data, however, show dissimilar trends in which Peth $^{13}$C labeling is non-linear and the PtdE data are linear (Fig. 7). Further incorporation of label from PtdE into PtdC by the PEMT pathway leads to a linear trend in the C2-PtdC data (Fig. 8). Since these phospholipid pools (C2-PtdE and C2-PtdC) acquired label at a constant linear rate, the data suggest that the flux for the precursor pool (Peth) must also be constant. Thus Fluxes C, D, and E in Table 2 were held constant, even in Model-uf. The non-linear data for C2-Peth were fit by upregulating the flux between the Peth fast/slow pools (Flux D’) and then allowing a two-way exchange for this metabolite so the fast and slow pool sizes remained constant. The physiological significance of these data is not clear. The differences observed between PtdC and PtdE de novo synthesis, however, may reflect differences in
regulatory control of these pathways under the conditions used in this study. It appears that PtdE biosynthesis is maintained at a constant flux while de novo PtdC biosynthesis may be more highly regulated. The rate-controlling enzymes in these pathways, namely CCT and ECT, show differences in subcellular distribution and other regulatory factors ([24,30], and references therein) which may account for differences we observed in the pathway kinetics. Activation of CCT involves translocation and association with lipids and/or membranes, whereas the involvement of such processes in the regulation of ECT is less clear. Such evidence suggests that the two pathways are under independent metabolic control.

Model-uf provides a good fit of the experimental data (Figs. 6–8) and predicts that the rate of PtdE synthesis via the CDP-ethanolamine pathway exceeds the rate of PtdC synthesis via the CDP-choline pathway (Fluxes D and B, Table 2). The PEMT flux is about 40% of the PtdC ‘average’ flux and about 14% of the PtdE flux. Even in comparison to the maximum flux for the CDP-choline pathway (0.70 µmol/h/g), PtdE biosynthesis is 1.5 times greater. If we assume an average flux for the CDP-choline pathway of 0.35 µmol/h/g (the midpoint of the calculated range), then the CDP-ethanolamine pathway is about 3 times as fast. This result is contradictory to a study by Sundler et al. [3] in which rats were given an intraportal injection of radiolabeled choline plus ethanolamine and the rate of synthesis for PtdC was shown to exceed PtdE by 3 to 1. In our study, however, the substrates were infused in equimolar amounts (0.5 µmol/min) while the previous study used a single bolus injection of 0.5 µmol choline and 0.08 µmol ethanolamine. It is uncertain whether these differences in concentrations could account for the discrepancy. As mentioned previously, however, others have shown that the rate of PtdE synthesis is dependent upon the ethanolamine concentration [41,42]. In comparing actual rates it is interesting to note that Sundler et al. showed that rates of PtdC and PtdE synthesis were 4–5 times greater when choline and ethanolamine were administered together versus each substrate given separately [1–3]. Our rate of synthesis for PtdE (Flux D = 1.04 µmol/h/g liver) is within the range of rates reported by Sundler et al. (0.42 µmol/h/g liver when ethanolamine is given alone, and 2.1 µmol/h/g liver when given together with choline), but our value for PtdC synthesis (maximum for Flux B = 0.70 µmol/h/g liver) is much lower than that reported by Sundler et al. (1.5 µmol/h/g liver when choline is given alone, and 6.4 µmol/h/g liver when given together with ethanolamine); literature values were converted to comparable units assuming a liver weight of 10 g. An interesting interpretation concerns the possibility of competition between choline and ethanolamine. In other words, does ethanolamine and PtdE synthesis act to suppress PtdC synthesis? Consistent with this interpretation is the idea that the first enzyme in the pathways, choline/ethanolamine kinase, may consist of various protein isoforms [23]. One isoform is known to possess activity for both choline and ethanolamine substrates. Since the kinase activity resides in the same protein, the affinity for substrate may be of a competitive nature such that the availability of ethanolamine affects the affinity for choline, or vice versa. Choy et al. showed that ethanolamine competitively inhibited choline uptake and the incorporation of labeled choline into PtdC in the isolated hamster heart ([46] and references therein). More recently Jackowski et al. have identified a mammalian ethanolamine-specific kinase and suggest that this kinase activity may play a role in controlling PtdE biosynthesis [42]. Our experimental data and model simulations show that the flux from ethanolamine to Peth (Flux C) exceeds the flux from choline to Pcho (Flux A) at time points < 60 min (Table 2). The mid-range for Flux A in Model-uf is about one-half of the value for Flux C; and for Model-cf, Flux A is about one-third of Flux C. This indicates that ethanolamine uptake is approx. 2–3 times faster than choline for phospholipid biosynthesis. This is consistent with the possible differences in kinase activity for these substrates. Also, it is important to note that our results clearly show that choline was also used for betaine synthesis as discussed below.

4.5. Betaine (choline oxidation)

We found that choline was also diverted towards oxidation yielding betaine, as previously observed in vivo [1] and in rat hepatocytes [47]. The betaine NMR signal was undetectable prior to choline administration. It was also undetectable in spectra ac-
quired 60 min after infusion with unlabeled choline and ethanolamine. Thus the betaine NMR signal intensity represents a $^{13}$C enriched betaine pool. We were not able to measure the betaine concentration or $^{13}$C EF since this molecule does not contain a phosphorus atom and it was not detectable at natural abundance $^{13}$C concentrations. Thus, we cannot compare the flux from choline to betaine with that from choline to Pchο. The betaine signal intensity, however, was observed to increase linearly following infusion of $^{13}$C-labeled choline, but phosphorylation of choline showed a different trend, as the $^{13}$C label incorporation into Pchο was non-linear and showed a distinct lag over time. Although we did not measure the liver betaine concentration (as was done for Pchο), the NMR data suggest a steady increase in the betaine pool size and/or $^{13}$C enrichment of the pool. It is reasonable to assume that the liver betaine pool is insignificantly small prior to choline infusion and, consequently, the changes observed in the NMR data for betaine should reflect the label enrichment of the precursor pool (intracellular choline). Since, however, betaine synthesis occurs in the mitochondria and requires a specific transporter for choline, the pool of choline that is available to phosphorylation is different than that which is available for oxidation. Thus it is not surprising that the NMR data for Pchο and betaine are different. Others have shown that the uptake of choline by rat liver mitochondria results in a linear accumulation of betaine in the medium [48]. This result is similar to our observations for liver betaine.

5. Conclusions

These studies demonstrate the unique capabilities of NMR and isotopic labeling to examine the specific metabolic pathways of phospholipid biosynthesis in vivo. Kinetic analyses of the data indicate that the CDP-choline and CDP-ethanolamine pathways are channeled processes. We also provide, for the first time, experimental evidence that the PEMT pathway for conversion of PtdE to PtdC is channeled. When rats are presented with equimolar amounts of choline and ethanolamine, liver PtdE biosynthesis occurs at a constant rate which is 2–3 times the rate of PtdC synthesis by the CDP-choline pathway. Of the newly synthesized PtdC, 70% was derived by the CDP-choline pathway and 30% was through the PEMT pathway. The rate of PtdC synthesis through the PEMT pathway was about one-tenth the rate of PtdE biosynthesis. Also, the non-linear labeling of several intermediate metabolites suggests that the metabolic fluxes may not be constant during the experimental time course.

The current studies provide no information about the contribution of serine or phosphatidylserine metabolism to the pools of PtdC and PtdE. Further studies using $^{13}$C-labeled serine may help to address these issues. NMR techniques of the type demonstrated herein can also be used to examine phospholipid metabolic pathways in various pathophysiologies.

Acknowledgements

A portion of this work was sponsored by the Air Force Office of Scientific Research, Air Force Systems Command, USAF, under Grant or Cooperative Agreement F49620-1-0180.

References
