Phosphorylethanolamine – the major constituent of the phosphomonoester peak observed by ^{31}P-NMR on developing dog brain

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^{31}P-NMR spectra of newborn dog brains exhibit a prominent phosphomonoester (PME) peak (6.78 ± SD. 0.05 ppm from phosphocreatine peak), similar to those of human neonates. Studies were undertaken to identify the chemical constituents of this peak. Brains of puppies were funnel frozen for methanol–HCl–perchloric acid extraction after in vivo ^{31}P-NMR spectra were taken. The pK of the major component of the PME region in the NMR spectrum of extract was 5.4, corresponding to that of phosphorylethanolamine (PEt). Addition of PEt increased the major peak on the PME region over a wide range of pH, while addition of phosphorylcholine or ribose 5-phosphate yielded distinct peaks. We suggest that the major constituent of phosphomonoester peak of ^{31}P-NMR spectra of newborn dog brain is phosphorylethanolamine. Biochemical mechanisms relevant to changes of phosphorylethanolamine during brain development are discussed.

^{31}P-NMR spectroscopy in vivo *Phosphorylethanolamine* *Brain development* *Phosphomonoester* *Sugar phosphate* *Phospholipid synthesis*

1. INTRODUCTION

The presence of phosphomonoesters (PME) was discovered in tumor tissues as early as 1937 [1]. In 1939 these compounds were found in the small intestines of rabbits and pigs [2]. Ten years later authors in [3] reported the presence of phosphorylethanolamine (PEt) in 23 organs and various malignant tumors of rat. Then it was shown [4] that the PEt concentration decreased during postnatal development of mammalian brain. This observation lay fallow until ^{31}P-NMR studies, particularly [5,6], called attention to the existence of an intense PME peak in the in vivo ^{31}P-NMR spectra of human neonate brains. The intensity of the PME peak appears somewhat diminished in an 8-year-old child [7] and decreases further in adult humans (J.S. Leigh and C.H. Barlow, Biophysics Today (July 22, 1983) Authors in [8,9] have also observed similar high levels of PMEs in tumor tissues, particularily in neuroblastoma [8].

The identification of compounds which contribute to the PME peak of in vivo ^{31}P-NMR spectra is difficult, since ^{31}P resonance peaks of numerous phosphomonoesters are reasonably close [9–11] and therefore overlap. It was suggested [5] that ribose 5-phosphate was the major component of this peak in the ^{31}P-NMR spectra of the human neonate brain. Among the PMEs giving resonance peaks in this region are sugar phosphates such as glucose 6-phosphate, ribulose 5-phosphate, xylulose 5-phosphate, fructose 1,6-diphosphate, erythrose 4-P, besides ribose 5-phosphate; however, the total concentration of these compounds is generally not more than 0.5–0.6 mM [12–14] in brain. Other PMEs such as PEt and phosphorylcholine also have resonance peaks in this region, and their concentration ranges from

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2–4 mM depending on species and age [15]. Therefore, our hypothesis was that one of these compounds could be the major contributor to this resonance peak. Preliminary experiments show that the in vivo ^{31}P-NMR spectra of brains of human neonates and newborn dogs are similar. Therefore, puppies can serve as a convenient model for studying the chemical nature of the PME region. It will be demonstrated that the major constituent of the PME peak of ^{31}P-NMR spectra brains of newborn dog is PEt.

2. MATERIALS AND METHODS

2.1. *In vivo ^{31}P-NMR of puppy brain*

Experiments were carried out on 17-day-old puppies, anaesthetized with Inovar-Vet (0.2 ml/kg). In vivo ^{31}P-NMR spectra were taken on a 1.5 T 12 in diameter bore Oxford magnet equipped with a Phosphoenergetics spectrometer. A two-turn 3.8 cm diameter copper coil was tuned and matched to 24.5 MHz and placed on the skull after the skin and muscle were removed. Acquisition parameters were nominal 90° flip angle; repetition rate 0.25 Hz, total observation time 16 min. Chemical shifts are reported as ppm from PCr.

2.2. *Preparation of acid extracts of brain*

Brains of puppies ($n=3$) were funnel frozen with liquid nitrogen [14] following the in vivo NMR spectrum. The brains were carefully removed from the cerebral cavity and pulverized at −195°C in liquid nitrogen. Methanol–HCl–PCA (perchloric acid) extraction was performed as described in [16]. The extract was passed through a Chelex column to lower the concentration of alkaline earth and transition metal ions. The extract was then lyophilized and dissolved in 2.5 ml of 20% D_2O before NMR analysis.

2.3. *^{31}P-NMR analysis of extracts*

^{31}P-NMR spectra were obtained using a Bruker WH-360 NMR spectrometer operating at 145 MHz for phosphorus. Acquisition parameters were: pulse width, 45 μs; repetition rate, 0.4 Hz; sweep width, 5000 Hz; acquisition time, 1.6 s; line broadening, 0.5 Hz. Chemical shifts are reported as ppm from 85% orthophosphoric acid using glycerolphosphorylcholine ($\delta=0.49$) as an internal standard. PCr is −2.39 ppm from orthophosphoric acid.

2.4. *Identification of the resonance peaks*

Identification of the major PME resonance peak in brain extracts was made according to the following protocol: (i) NMR analysis using a library of resonance peak positions according to the existing data in the literature [9–11,17]. (ii) Determination of the pH dependence of the chemical resonance shift in the extract for the peaks of interest and determination of the pK. (iii) Addition of known phosphate compound to the extract to determine whether the resonance peak originating from the added substance is superimposed on the peak of interest at several different pH values.

Further evidence that the compound identified can be attributed to the peak of interest in vivo was provided by pH titrations of possible candidates in a solution resembling the composition of whole brain. The concentration of the constituents of the model solution were as follows: NaH_2CO_3, 10–15 mM; Na_2HPO_4, 10–15 mM; $MgCl_2$, 8 mM; KCl, 90 mM; ATP, 5 mM; PCr, 10 mM. The pH-dependent chemical shift curve of the following substances were determined: ribose 5-phosphate, glucose 6-phosphate, PEt, PCh and P_i. The pH of the solution was changed by adding 5 N KOH or 4 N HCl. ^{31}P-NMR spectra were taken at 37°C on a Bruker 200 CXP NMR spectrometer operating at 80.98 MHz for phosphorus and 200 MHz for proton. Acquisition parameters were: 45° flip angle; repetition rate, 0.4 Hz; 60 repetitions; sweep width, 5000 Hz.

3. RESULTS AND DISCUSSION

Fig.1 is a typical in vivo ^{31}P-NMR spectrum of a puppy brain illustrating its close resemblance to spectra of human neonates [5,6]. The chemical shift of the prominent phosphomonoester peak was 6.78 ± SD 0.05 ppm, that of the P_i was 5.05 ± SD 0.11 ppm from PCr ($n=5$). The ^{31}P-NMR spectra of puppy brain showed a PME/PCr ratio of 1.99 ± SD 0.25 and PME/ATP ratio of 0.95 ± SD 0.23. ^{31}P-NMR spectra of adult dog brain also exhibit a prominent PME peak (fig.2, table 1) equivalent to approx. 2 mM concentration.

The results of the pH dependence of the chemical shifts of glucose 6-phosphate, ribose 5-phos-

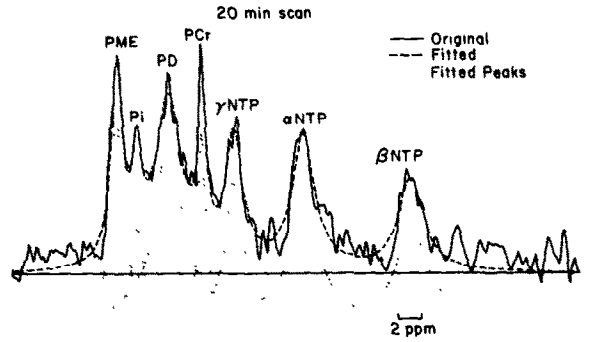

Fig.1. ^{31}P-NMR spectrum of a 17-day-old puppy brain. PME, phosphomonoesters; P_i, inorganic phosphate; PD, phosphodiesters; PCr, phosphocreatine; αNTP, βNTP, γNTP, α,β,γ, phosphate of nucleotide triphosphates. The fitted spectrum and peaks were determined as in [8]. The fitted peaks are shifted down.

phate, PEt, PCh and P_i in the model solution for whole brain (fig.3) show that, when the chemical shift of P_i from PCr is 5.00 ppm, that of PEt is 6.8, which is similar to the chemical shift of the PME peak in the in vivo spectrum. However, at this pH, glucose 6-phosphate would be downfield while ribose 5-phosphate and PCh are upfield of PEt (fig.3). Fig.4 shows a spectrum of a PCA extract of puppy brain, the in vivo spectrum of which is shown in fig.1. The dominant peak in the PME region is 4.5 ppm from 85% orthophosphoric acid

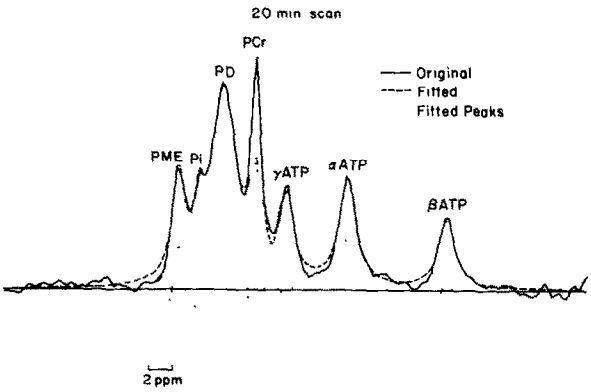

Fig.2. ^{31}P-NMR spectrum of adult dog brain. Peak identification: see fig.1. Spectra were taken on a Phosphoenergetics 12 in NMR system using a 4-cm diameter single turn surface coil. Acquisition parameters repetition rate, 0.25 Hz; flip angle, nominal 90°; total observation time 20 min.

Table 1

Relative peak areas from in vivo ^{31}P-NMR spectra of brains of various species

		PME / P_i	PME / PCr	PME / ATP	PCr / PI	PCr / ATP
I	Newborn dog	2.21	1.99	0.95	1.13	0.48
II	Human neonate	2.78	2.14	1.5	1.3	0.7[a]
III	Adult dog	1.86	0.74	0.80	2.71	1.14[b]
IV	Adult gerbil	1.14	0.54	1.00	2.21	1.75[c]

[a][6]
[b]Also [18]
[c][19]

at pH 7.5. The pH titration of this resonance peak (fig.5) in the extract resulted in a pK_a of 5.4 which corresponds closely to the pK_a of PEt [9,11]. Addition of PEt to the extract resulted in peak superimposed on this peak at pH 7.5 (fig.6) and over a wide range of pH values (fig.5). Addition of ribose 5-phosphate to this sample resulted in a superimposed peak at 4.5 ppm at pH 7.5; however, at pH 6.6 the ribose 5-phosphate peak shifted to 3.7 ppm while the composite peaks of added PEt and the

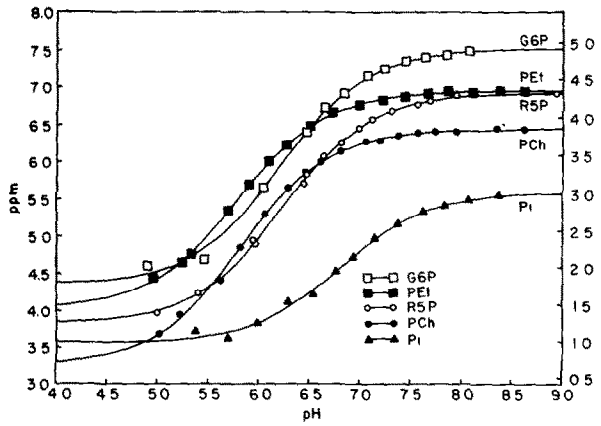

Fig.3. Dependence of chemical shifts of PEt, phosphorylcholine (PCh), ribose 5-phosphate (R5P), glucose 6-phosphate (G6P) and inorganic phosphate (P_i) on pH. Composition of medium is described in section 2. Chemical shift expressed relative to both orthophosphic acid (right scale) and PCr (left scale), respectively. Measurements were made at 37°C.

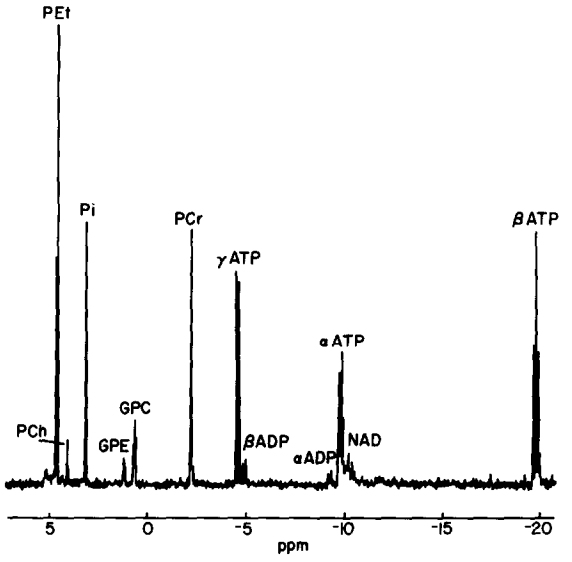

Fig.4. ^{31}P-NMR spectrum of puppy brain extract. This spectrum is a result of 3000 accumulations (conditions in text). Assignment of peaks in the PME region made in accordance with experimental results. PEt, phosphorylethanolamine; PCh, phosporylcholine; GPC, glycerolphosphorylcholine; GPE, glycerolphosphorylethanolamine.

major PME peak in the brain extract remained superimposed at 4.2 ppm. Addition of phosphorylcholine resulted in peak upfield from the peaks of interest at wide range of pH values (fig.5,6). Based on the above data, we concluded that the peak at 4.5 ppm in the brain extract arises from PEt. Since at the physiological intracellular pH, as determined by the chemical shift of P_i, the chemical shift of PEt in our model solution (fig.3) and in vivo (fig.1), is practically the same, we suggest that the prominent peak in PME region of ^{31}P-NMR spectra of in vivo puppy brain originates mainly from PEt. Furthermore, because the chemical shift of the PME peaks in newborn infants is 6.72 ± SD 0.11 [5], we suggest the major component of this peak is PEt. The chemical shift of PME peak on dog brains is 6.71 ± SD 0.09 (that of P_i = 4.84 ± SD 0.11) (see also [18]) and on gerbil brain it is 6.76 ± SD 0.02 (P_i = 4.9 ± SD 0.04) ppm [19]; therefore, it seems probable that the PEt of these species also contributes to the PME peak.

The fact that PME peak is less prominent in adult dogs [18] (fig.2) than in puppies corresponds to the well established fact that PEt concentration

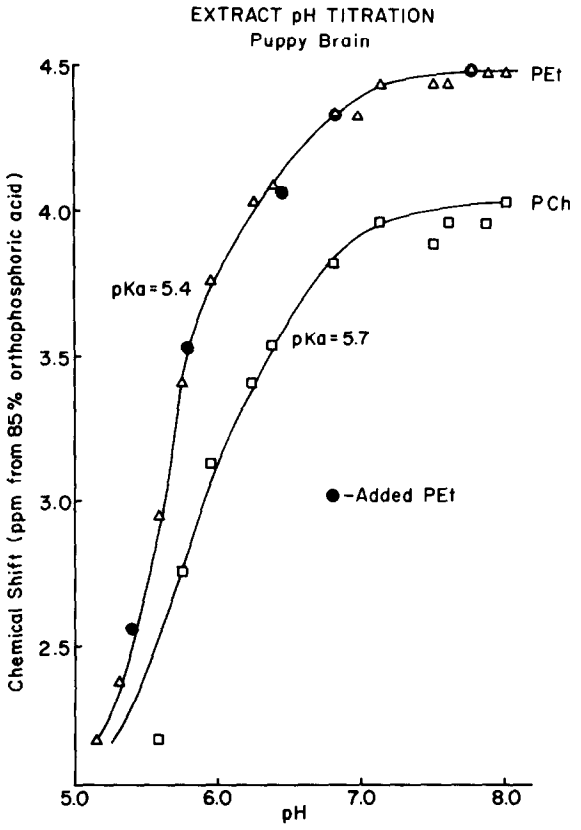

Fig.5. pH dependence of chemical shift of PEt (△—△) and PCh (□—□) in the puppy brain extract. pK_a values were determined to be 5.4 and 5.7 for PEt and PCh, respectively. (●—●) added PEt.

of the brain decreases during maturation in dogs [20]. The exact biochemical mechanism explaining the changes in concentration of PEt is not well described in the literature. Experimental data suggest that the cytidyltransferase reaction is the rate limiting step [21] in the enzymatic reactions involved in the de novo synthesis of phosphatidylethanolamine, although the ethanolamine-kinase is also in disequilibrium [22]. Consequently, an increase in the rate of the cytidyltransferase reaction should lead to a decrease of PEt and an increase in phosphatidylethanolamine. Indirect evidence suggests that this hypothesis holds true in vivo. The synthesis of phosphatidylcholine (lecithin) is catalysed by similar enzymes and involves analogous steps to that of phosphatidylethanolamine synthesis. The kinase reaction varies little, while the activity of cytidyltransferase and phosphoryl-

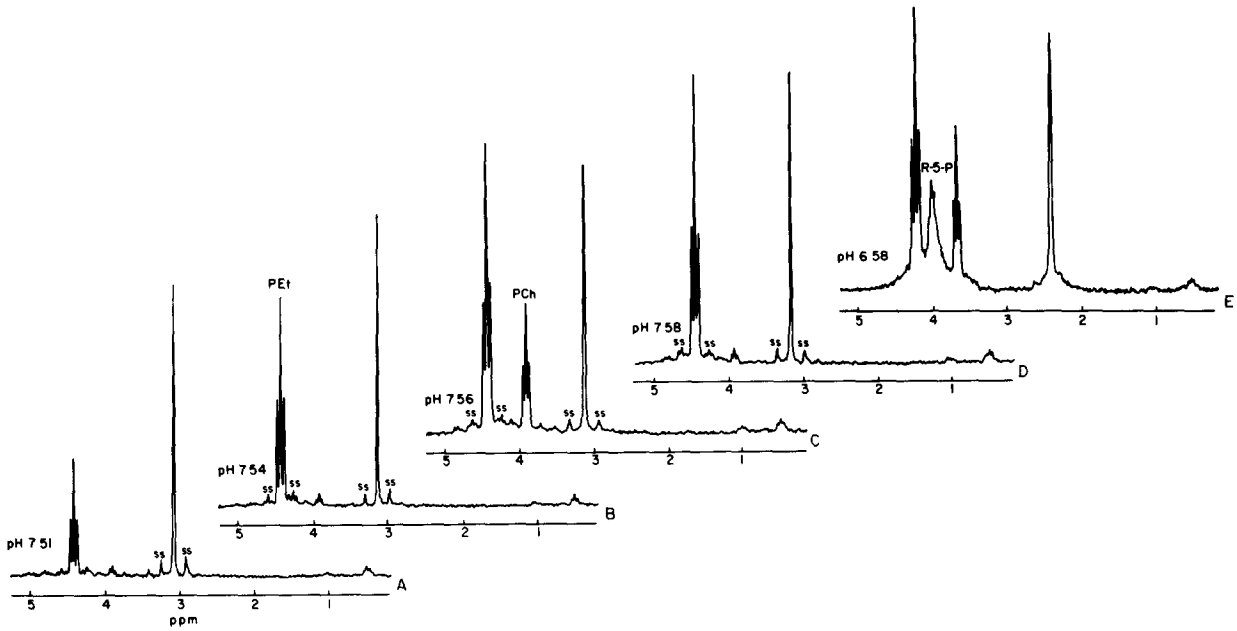

Fig.6. PME region of puppy brain extract before and after addition of known compounds. A. Before additions, pH 7.51. B. With added PEt, pH 7.54. C. With added PCh, pH 7.56. D. With added R-5-P, pH 7.58. E. Same as D except at pH 6.58 (with PCh).

choline transferase increases in developing brain post partum [23]. Also, the phosphatidylethanolamine concentration increases in brain during maturation [24]. This pattern corresponds to the one suggested above. These results show that with careful analysis of ^{31}P-NMR spectra of brain, in vivo ^{31}P-NMR could be useful in studying metabolism of phosphoglycerides and the maturation of the brain.

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