QUANTITATIVE ROLE OF BASE EXCHANGE IN PHOSPHATIDYLETHANOLAMINE SYNTHESIS IN ISOLATED RAT HEPATOCYTES*

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1. Introduction

The biosynthesis of phosphatidylethanolamines from ethanolamine proceeds via formation of phosphoryl- and CDP-ethanolamine [1]. In cell-free systems from liver [2–4] and brain [5,6] ethanolamine is also incorporated by a calcium-stimulated exchange with the base moiety of pre-existing phospholipids. The rate and physiological importance of this base exchange in intact cells is not known although results obtained in vivo gave no indication that it is quantitatively important in rat liver [7]. In the present study the question has been further investigated using enzymatically suspended rat hepatocytes.

The relative rate of incorporation of labeled ethanolamine via base exchange and the CDP-ethanolamine pathway have been evaluated in two different ways. Firstly, it was assumed that ethanolamine incorporation remaining after total inhibition of energy-dependent phosphatidylethanolamine synthesis by cyanide and fluoride is due to base exchange. Secondly, advantage was taken of the previous finding that addition of albumin-bound oleic or linoleic acid changes phosphatidylethanolamine synthesis via CDP-ethanolamine so that high proportions of dioleoyl- or dilinoleoyl-phosphatidylethanolamine are formed [8,9]. Under such conditions a comparison of the labeling of different phosphatidylethanolamines from [^{32}P]phosphate (incorporated only via CDP-ethanolamine) and [^{3}H]ethanolamine (in addition incorporated by base exchange) enabled determination of the relative role of the two pathways.

Results obtained by the two approaches agreed very well and showed that at a physiological concentration of ethanolamine 8–9% of its total incorporation into phosphatidylethanolamines can be attributed to the exchange reaction but that this proportion increases to 30–40% when its concentration is raised twenty-fold

2. Materials and methods

[2-^{3}H]Ethanolamine (320 mCi/mmole) was obtained from The Radiochemical Centre, Amersham and was purified by thin-layer chromatography on Silica gel G (Merck AG, Darmstadt) (developing solvent: ethanol–2% NH_4OH 1:2 by vol). [^{32}P]Phosphate was obtained from AB Atomenergi, Studsvik, Sweden. Bovine serum albumin (Serva, Heidelberg) was delipidated [10] and dialyzed and oleic or linoleic acid was complexed to the albumin [11]. Male Sprague–Dawley rats (Anticimex AB, Sweden) weighing 200–250 g and fed a balanced diet were used for the preparation of hepatocytes [12,13].

The incubations contained approx. 2×10^6 cells (1.0–2.3 mg protein) in a total volume of 0.5 ml Hanks' solution [14] buffered with 10 mM HEPES* (pH 7.4) and were performed in siliconized 25 ml Erlenmeyer flasks as previously described [8] except

* A preliminary account of this work was presented at the 9th Int. Congr. of Biochem., Stockholm, 1973.

* HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

for the following modifications. In experiments where total and energy-independent incorporation of [^{3}H]-ethanolamine was determined, incubations containing cells and nonradioactive additions with or without 20 mM sodium cyanide and 20 mM sodium fluoride were incubated for 30 min prior to the addition of [^{32}P]phosphate (10–20 μCi). Since the incorporation of [^{32}P]phosphate into phosphatidylethanolamines showed an initial time lag (fig. 1) [^{3}H]ethanolamine (1–3 μCi; usually 10–15 nmoles) was added 15 min after [^{32}P]phosphate addition. Incubations with oleic or linoleic acid contained 2% albumin and 1.5–2.2 mM fatty acid and were started by the addition of [^{32}P]phosphate, followed 15 min later by [^{3}H]ethanolamine. Incubations were terminated 30 min after [^{3}H]ethanolamine addition unless otherwise stated and lipids were extracted and washed free of labeled precursors [9].

Phosphatidylethanolamines were isolated from the lipid extract by thin-layer chromatography on Silica gel H (Merck AG) with chloroform–methanol–water (70:30:5 by vol.) as developing solvent and were resolved into molecular species by argentation chromatography as *N*-benzoyl-*O*-methyl-derivatives [15].

Radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb instrument. Quenching was monitored with an automatic external standard. The correction curve for ^{32}P was obtained with freshly prepared ^{32}P-labeled phospholipids. The counting solution was either that of Bray [16] or Instagel (Packard)–toluene (1:1 v/v) as specified elsewhere [8].

3. Results and discussion

Fig. 1 shows that after an initial time lag for ^{32}P, the incorporation of [^{32}P]phosphate as well as of [^{3}H]ethanolamine was linear for about 30 min. Then the incorporation of [^{3}H]ethanolamine levelled off due to substrate depletion. The presence of albumin-bound oleic or linoleic acid did not significantly affect the total incorporation of labeled substrates into phosphatidylethanolamines under these conditions. When the cells were preincubated with cyanide and fluoride prior to the addition of isotopes, phosphatidylethanolamine synthesis from [^{32}P]phosphate was inhibited by more than 99% while part of the incorporation of [^{3}H]ethanolamine remained (fig. 1b). At 0.02–0.03 mM ethanolamine, concentrations similar to that in rat blood plasma (unpublished results), the energy-independent incorporation of [^{3}H]ethanolamine amounted to about 9% of that in incubations without cyanide and fluoride (table 1). It is likely that this incorporation occurred via base exchange since it was strongly inhibited by disodium-EDTA or serine (table 1), which characterizes ethanol-

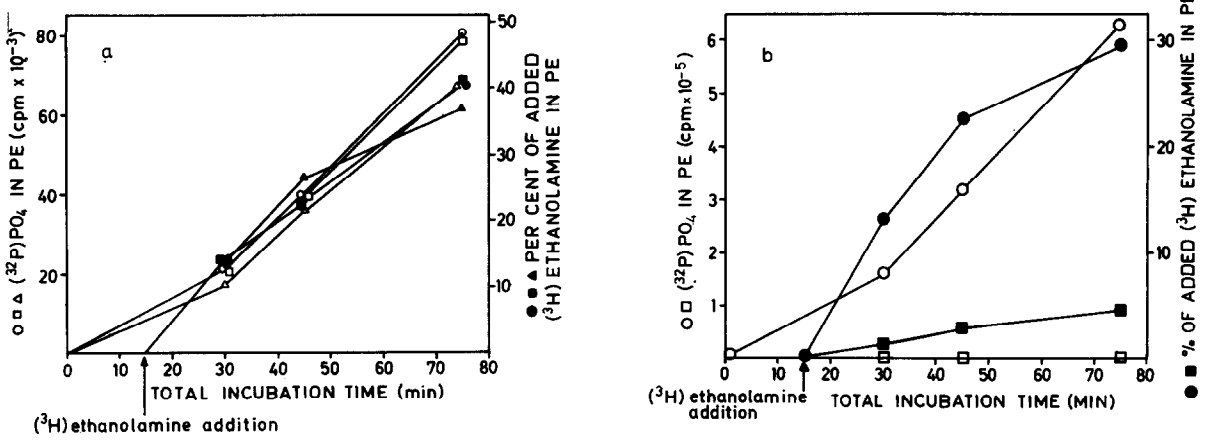

Fig. 1. Time course of phosphatidylethanolamine (PE) synthesis from [^{32}P]phosphate (○, △, □) and [^{3}H]ethanolamine (●, ▲, ■) in isolated hepatocytes. In fig. 1a the cells were incubated in 2% bovine serum albumin without fatty acid (○, ●), with 1.9 mM oleic acid (△, ▲) or 2.2 mM linoleic acid (□, ■). In fig. 1b the cells were preincubated for 30 min prior to [^{32}P]phosphate addition; in the absence (○, ●) or presence (□, ■) of 20 mM NaCN and 20 mM NaF.

Table 1
Contribution from base exchange to the incorporation of [^{3}H]ethanolamine into phosphatidylethanolamines. Effect of serine and EDTA

Addition (mM)	Incorporation by base exchange (%)	
	A	B
None	8.8 ± 1.8 [4]	8.2 ± 1.6* [6]
DL-Serine (0.5 or 1.0)	0.4 – 1.2 [2]	0.0 – 1.7** [3]
Na_2 EDTA (3.0)	0.0 – 1.1 [3]	0.0 – 1.0 [2]

* Mean ± S.D. (number of separate experiments).
** Range (number of separate experiments).

Data in column A represent [^{3}H]ethanolamine incorporation in cells preincubated with NaCN and NaF (20 mM each) expressed as per cent of incorporation in uninhibited controls. The presence of NaCN and NaF lowered [^{32}P]phosphate incorporation into phosphatidylethanolamines by more than 99%.

Data under B were calculated from the percentage distribution of [^{3}H]ethanolamine and [^{32}P]phosphate among phosphatidylethanolamines after incubation with linoleic acid. Isotope in the dienoic–dienoic (DD) species was considered exclusively incorporated via CDP-ethanolamine. The percentage contribution from base exchange was then determined as:

$$\left[1 - \frac{\%\text{ of }^3\text{H in DD}}{\%\text{ of }^{32}\text{P in DD}}\right] \times 100$$

[^{3}H]Ethanolamine was present at 0.02–0.03 mM.

amine exchange in different cell-free systems [2,3,5,6, 17,18].

In order to decide whether the simultaneous operation of phosphatidylethanolamine synthesis via CDP-ethanolamine might affect the rate of the exchange reaction with ethanolamine, conditions were sought under which the incorporation of ethanolamine via the latter pathway could be determined without inhibiting the former. By incubating the hepatocytes with albumin-bound oleic or linoleic acid de novo glycerolipid biosynthesis is changed so that high proportions of molecular species containing only the added fatty acid are formed (fig. 2; ref. [8,9]). If in the present experiments [^{3}H]ethanolamine and [^{32}P]phosphate were both incorporated only via CDP-ethanolamine, one would expect their distribution among the molecular species of phosphatidylethanolamine to be identical. Fig. 2 shows that the addition of oleic or linoleic acid affected the distribution of the two isotopes in a similar, but not identical, way. The difference between the distribution of the isotopes was essentially the same whether oleic or linoleic acid was added (fig. 2) and was constant at different times of incubation. The results therefore indicate that part of the labeled ethanolamine found in phosphatidylethanolamines had been incorporated by a mechanism other than the CDP-ethanolamine pathway responsible for [^{32}P]phosphate incorporation. If it is assumed that the distribution of this part of the labeled ethanolamine among molecular species of phosphatidylethanolamine is not significantly changed by the fatty acid additions, it may be calculated that this incorporation accounts for about 8% of the total [^{3}H]ethanolamine incorporation at 0.02–0.03 mM ethanolamine (table 1). This incorporation

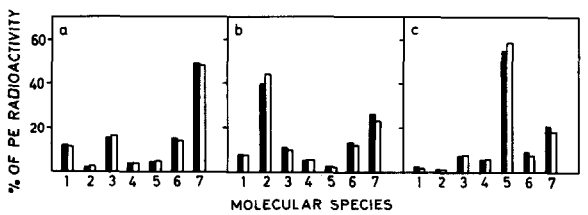

Fig. 2. Distribution of [^{3}H]ethanolamine and [^{32}P]PO_4 among phosphatidylethanolamine (PE) species of different unsaturation. Incubations were carried out without fatty acid addition (a), with 1.9 mM oleic acid (b) or 2.2 mM linoleic acid (c). 1 = saturated–saturated, 2 = saturated–monoenoic, 3 = monoenoic–monoenoic *plus* saturated–dienoic, 4 = monoenoic–dienoic, 5 = dienoic–dienoic, 6 = saturated–tetraenoic and 7 = saturated hexaenoic species. Total incubation time was 45 min. Open bars –^{32}P, filled bars –^{3}H.

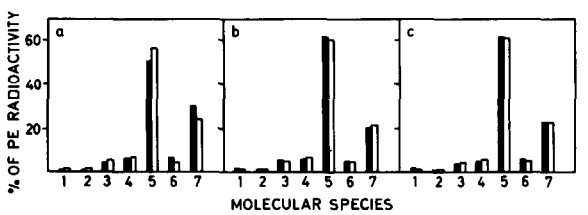

Fig. 3. Effect of serine and disodium-EDTA on the distribution of [^{32}P]phosphate and [^{3}H]ethanolamine among molecular species of phosphatidylethanolamine (PE). The incubations contained 1.5 mM albumin-bound linoleic acid and in addition 1 mM DL-serine (b) or 3 mM Na_2EDTA (c). Total incubation time was 45 min. Symbols as in fig. 2.

Table 2
Incorporation of ethanolamine into different phosphatidylethanolamines by base exchange

Phosphatidylethanolamine species	Percentage distribution		
	Isolated rat hepatocytes (present study)		Rat liver microsomes (ref. [3])
	A	B	
Saturated–monoenoic	2.4 ± 0.2	5.8 ± 5.1	5.4 ± 3.0
Monoenoic–monoenoic *plus* saturated–dienoic	12.1 ± 2.3	7.4 ± 5.0	13.9 ± 1.7
Monoenoic–dienoic	1.7 ± 0.2	0.2 ± 0.3	–
Dienoic–dienoic	2.0 ± 0.3	–	–
Saturated–tetraenoic	35.6 ± 3.1	35.7 ± 6.7	43.9 ± 5.5
Saturated–hexaenoic	46.2 ± 5.3	50.9 ± 7.0	36.8 ± 6.4
	[4]	[10]	[6]

The data on isolated hepatocytes were obtained from incubations containing cyanide and fluoride (column A) or from incubations with linoleic acid (column B). The latter data were calculated as follows: The percentage of ^{3}H in each molecular species of phosphatidylethanolamine was divided by the ratio $\frac{\%\ ^{3}H \text{ in DD}}{\%\ ^{32}P \text{ in DD}}$, where DD represents the dienoic–dienoic species. The excess of the corrected ^{3}H versus ^{32}P (% ^{3}H–% ^{32}P) in each species, which was attributed to incorporation by base exchange, was summed up and its distribution was calculated. The figures are means ± S.D. (number of samples).

of ethanolamine, like the energy-independent one, was strongly inhibited by disodium-EDTA and serine (fig. 3, table 1) indicating that it was due to calcium-stimulated base exchange. Furthermore, when the incorporation of ethanolamine into different phosphatidylethanolamines by base exchange was determined by any of the present methods, the results were in reasonable agreement with characteristics of the calcium-stimulated incorporation of ethanolamine in rat liver microsomes (table 2). Substitution of oleic acid for linoleic acid gave analogous results to those in table 2. The latter finding supports the assumption that the fatty acid composition of the phospholipid substrates in the exchange reaction is significantly changed during these short term experiments.

When the concentration of [^{3}H]ethanolamine in the incubation medium was raised, the difference in distribution of [^{3}H]ethanolamine and [^{32}P]phosphate among molecular species of phosphatidylethanolamine was augmented (fig. 4), indicating that an increasing proportion of the labeled ethanolamine in phosphatidylethanolamines had been incorporated

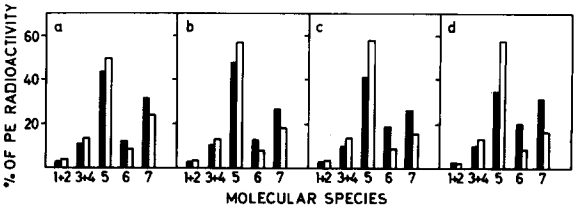

Fig. 4. Effect of ethanolamine concentration on the distribution of [^{32}P]phosphate and [^{3}H]ethanolamine among molecular species of phosphatidylethanolamine (PE). Experimental conditions as in fig. 3a, except that the final concentration of ethanolamine was 0.03 (a),).06 (b), 0.17 (c) and 0.41 mM (d), respectively.

via base exchange (fig. 5). Also the energy-independent proportion of the total [^{3}H]ethanolamine incorporation increased at higher concentrations of ethanolamine (fig. 5). This increase in the relative rate of the exchange reaction is consistent with unpublished data which show that the CDP-ethanolamine pathway is saturated already of 0.05 mM ethanolamine.

The presence of disodium-EDTA, which inhibited ethanolamine incorporation via base exchange by

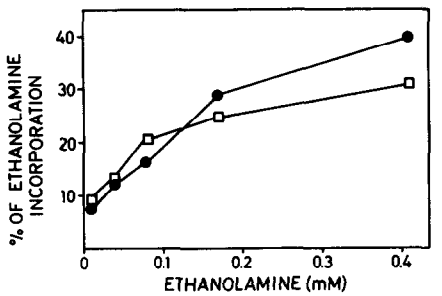

Fig. 5. Effect of the concentration of [^{3}H]ethanolamine on the percentage contribution from base exchange to the total incorporation of [^{3}H]ethanolamine into phosphatidylethanolamines. The contribution from base exchange was determined as the energy-independent incorporation of [^{3}H]ethanolamine (□) or calculated (see table 1) from data obtained after incubation with linoleic acid (●).

about 90% (table 1) also lowered the incorporation via CDP-ethanolamine but only by 25–35%. Since also the latter pathway depends on the presence of a divalent cation, namely Mg^{2+} [19,20], the more drastic effect of EDTA on the exchange reaction raised the question whether this reaction in our system occurred mainly in a small population of damaged cells with abnormally high permeability. However, this seems unlikely since the energy-independent incorporation did not increase when the cells were broken by homogenisation in a Potter–Elvehjem Teflon-glass homogenizer.

The relevance of results obtained by the present approach to the situation in vivo was tested by determination of the relative rate of choline exchange. A previous study indicated that the rate of this reaction is about 1/20 of that of the CDP-choline pathway in intact rat liver [2]. In isolated hepatocytes the energy-independent incorporation of 0.04 mM [Me-^{3}H]choline was 4.5 and 4.9% of that in uninhibited control incubations in two separate experiments.

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