ENZYMIC SYNTHESIS OF PLASMALOGEN AND *O*-ALKYL GLYCEROLIPID BY BASE-EXCHANGE REACTION IN THE RAT BRAIN*

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1. Introduction

It has been demonstrated recently that a Ca^{2+}-dependent base-exchange system occurs in subcellular fractions of brain which can convert *in vitro*, at the expenses of endogenous phospholipid, labelled ethanolamine, serine, choline or other nitrogenous bases into EPG, SPG, CPG or other lipids [1–3]. Membranes from the endoplasmic reticulum of chick, rat and rabbit brains were found to possess the highest rate of incorporation, and the requirement for Ca^{2+} appeared to be absolute.

The reaction, which takes place also in non-nervous tissue (see [2]), is regarded as an exchange process, not requiring energy, between the bound-base of the exchanging membrane phospholipid and the free base [2, 3], and cannot be compared for several reasons with any of the known pathways of lipid synthesis.

On studying this system we obtained evidence that the greater part of the incorporated ethanolamine or serine (about 85%) was confined to labelled diacyl-GPE or diacyl-GPS, respectively; the remainder of the radioactivity was not accounted for. The experiments described here were aimed at examining whether alkenylacyl-GPE and alkenylacyl-GPS could also be produced by similar mechanisms. It is shown that the microsomal fraction from rat brain converts by base-exchange labelled L-serine or ethanolamine into the plasmalogen derivatives [1–3], and the reaction stem are similar to those described for the synthesis of the diacyl derivative [1–3], and the reaction requires Ca^{2+} as the only factor to obtain the exchange.

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It is also reported that a similar mechanism can convert free nitrogenous bases into alkali-stable and acid-stable phospholipids, probably alkylacyl derivatives.

2. Experimental

Male Sprague-Dawley rats (120–150 g) were used. Subcellular fractions in 2 mM-EDTA-containing medium were prepared from brain and further purified, as described previously [2]. The methods for assessing their purity are reported elsewhere [2]. Incubation conditions are described in the tables. The incubations were terminated by adding 9 vol of chloroform–methanol (2:1, v/v) followed by a brief agitation. After one more extraction and filtration, the extracts

Abbreviations:
EPG, ethanolamine phosphoglycerides;
SPG, serine phosphoglycerides;
CPG, choline phosphoglycerides;
diacyl-GPE, 1, 2-diacyl-*sn*-glycero-3-phosphorylethanolamines;
diacyl-GPS, 1, 2-diacyl-*sn*-glycero-3-phosphorylserines;
alkenylacyl-GPE, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphorylethanolamines;
alkenylacyl-GPS, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphorylserines;
alkylacyl-GPE, 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamines;
alkylacyl-GPS, 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylserines;
TLC, thin-layer chromatography;
monoacyl-GPE, 2-monoacyl-*sn*-glycero-3-phosphorylethanolamines.

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were treated and purified as reported elsewhere [2], and taken to dryness in a stream of N_2 (lipid extract).

The incorporation of radioactivity into lipid fractions was first examined by a two-step TLC procedure [4], which allows on a single chromatogram the complete separation of the main classes of phospholipids of nervous membranes. Lipid classes were identified by the use of purified markers. Alternatively, TLC on silica gel G plates (250 μm thickness) with chloroform–methanol–ammonia (70:30:5, by vol.) as solvent, was used.

Synthesis of plasmalogens was verified by the use of the following four methods:

Method (*a*): the lipid extract was resolved by two-dimensional TLC with cleavage of the alkenyl groups with HCl between runs [5]. This procedure separates the 2-monoacyl derivatives produced from plasmalogen from the mixture of alkylacyl and diacyl derivatives. Radioactivity and P content were examined as described below. Recovery for both P and isotope assay were 95–100% as checked with calibration curves with standard products of known radioactivity and P content.

Method (*b*): this procedure was used only for the analysis of labelled alkenylacyl-GPE. The purified lipid extract was fractionated by preparative TLC [6], and the EPG fraction, detected by radio-chromatographic scanning, scraped from the plate and eluted three times with chloroform–methanol (2:1, v/v), was taken to dryness under N_2 and examined for purity [6]. The residue (1.0–3.0 μmole P) was then iodinated [7] and chromatographed as described elsewhere [8] using chloroform–methanol–ammonia (70:30:5, by vol.) as the solvent. The separated alkenylacyl- and diacyl-GPE were examined as described below. Recovery for P and isotope content averaged 90–95%.

Method (*c*): microsomal lipids from a 10-fold-scaled up incubation mixture (about 350–400 μg P) were subjected to column chromatography according to Pries et al. [9], and the EPG- and SPG-containing fractions subjected to a selective hydrolysis procedure [9]. The final aqueous and organic phases were each diluted with methanol, and P and isotope contents determined. Small aliquots of each phase were than paper-chromatographed to separate the labelled phosphate esters (GPE andGPS), which were assayed for their P and isotope contents as reported below. The sum of these contents for the alkali-labile fraction, acid-labile fraction and alkali- and acid-stable fraction always approached the 100% of the original EPG + SPG content of the column eluate.

Method (*d*): column chromatography of total lipids (about 300–350 μg P) was carried out on Florisil (100–200 mesh, 0.08–0.15 mm, B.D.H., England), as described elsewhere [10, 11]. The procedure separates with accuracy the EPG from the SPG components, as checked by rechromatography on TLC [4, 5]. The fractions, concentrated to dryness under N_2, were treated as in method (*c*).

Incorporation of radioactivity into alkylacyl-glycerolipid was examined either (method *a*) by resolving the mixture of alkylacyl-GPE(GPS) and diacyl-GPE(GPS), separated from plasmalogens, by saponification and solvent partition [12, 13] and by assaying radioactivity and P on both aqueous and lower phases, or (methods *c* and *d*) by determining the P and isotope contents of the last lower phase lipids obtained after the alkaline and acid hydrolyses of separated EPG and SPG.

Protein and phospholipid-P were estimated as described elsewhere [2]. For P determinations, paper spots were directly digested [2], while silica gel spots were eluted three times each with chloroform–methanol (3:1, v/v), (2:1) and (1:1), and then the concentrated eluate examined. For radioactivity measurements, TLC spots were scraped from plates into counting vials, with addition of 0.2 ml of chloroform–methanol (2:1, v/v) and 10 ml of Instagel (Packard Instrument Co., Des Plaines, Ill., USA). Paper chromatography spots were cut out into vials, with addition of 1 ml of H_2O and 10 ml of Instagel. Quenching was monitored by the channels ratio method.

3. Results and discussion

When labelled ethanolamine was incubated with microsomes from rat brain, a low but reproducible incorporation of radioactivity into monoacyl-GPE was observed by separating the lipid classes by the two-step TLC procedure [4]. At this point, it was felt that monoacyl-GPE, clearly visible in this system between the separated phosphatidylcholine and sphingomyelin, could have been derived from alkenylacyl-GPE, owing to the acidic character of one of the two

Table 1
The incorporation of [1, 2-^{14}C]ethanolamine into EPG of rat-brain microsomes by the base-exchange reaction.

Experiments (methods of assay)*	Incorporation levels** Diacyl-GPE	Alkenyl-acyl-GPE	Alkylacyl-GPE
(*a*)	12.03 (86.1)	1.62 (11.6)	0.32 (2.3)
	13.02 (91.0)	0.89 (6.2)	0.40 (2.8)
(*b*)	9.18 (82.1)	1.98 (17.9)	–
(*c*)	70.0 (95.2) 69.4†	2.0 (2.8) 2.1†	1.48 (2.0)
	35.8 (94.9)	1.52 (4.0)	0.41 (1.1)
(*d*)	55.5 (94.2)	2.8 (4.8)	0.60 (1.0)

Brain microsomes (about 0.8–1.5 mg of protein for experiments *a* and *b*, and about 8–10 mg for *c* and *d*) were incubated at 37° in 40 mM Tris-HCl buffer, pH 8.5, 1.5 mM [^{14}C]ethanolamine (specific activity of 0.371 nCi/nmole) and 25 mM calcium chloride (all final conc.), added in that order. The final vol was 0.5 ml in experiments *a* and *b* and 3 ml in experiments *c* and *d*. Incubations were carried out for 30 min in heavy-walled, conical test-tubes, which were stoppered and shaken at about 100 strokes/min in a water-bath shaker. At the end of the incubations, the mixtures were treated as described in the text.

* See Experimental.

** For (*a*) and (*b*): nmole phospholipid synthesized × mg microsomal protein^{-1} × hr^{-1}; for (*c*) and (*d*): nmole phospholipid synthesized × hr^{-1}. Activity in experiments *c* and *d* has been expressed as nmole × hr^{-1}, because no strict relationships exist between activity and protein content at the higher range of protein concentration used here. Per cent of conversion into each phospholipid is shown in parentheses and is calculated on the sum of the labelled phospholipids, which always averaged 90–95% of the total incorporated radioactivity.

† After paper chromatographic separation of the corresponding labelled phosphoric ester, GPE (see method *c* in the text).

solvents used [4]. After these results, it was found necessary therefore to identify the labelled "plasmalogen" formed; this was done on the basis of the analyses described in the Experimental section.

Table 1 indicates, in this connection, that with any one of the four methods adopted for alkenylacyl-GPE separation and identification, appreciable incorporation of ethanolamine into this lipid occurs. The greater part of the incorporated ethanolamine (about 82–94%) is localized in diacyl-GPE, but a significant amount (about 4–12%) is found in alkenylacyl-GPE. The higher incorporation into alkenylacyl-GPE noted in experiments (*a*) and (*b*), as compared to experiments (*c*) and (*d*), is due most likely to the difficulty of isolating plasmalogens with complete recovery of the alk-1-enyl components by column chromatography [5, 14], whereas the separation–reaction–separation TLC procedure overcomes this difficulty [5]. On determining the incorporation after paper chromatography [9] of the labelled GPE, similar values of exchange were found (table 1), thus indicating that the label is confined to the base moiety of the lipid. Moreover, on performing experiments with method (*a*), not directly on the lipid extract, but on the column eluate obtained by methods (*c*) or (*d*), results exactly comparable to those of table 1 were obtained.

In order to confirm more precisely the results of table 1, dilution effects were studied after ethanolamine incorporation into EPG. Purified unlabelled diacyl-GPE or alkenylacyl-GPE [15] were added to the mixture in two different experiments soon after incubation. After extraction of phospholipids, the lipid extract was purified and resolved as specified in method (*a*) and the specific activities compared with those obtained without the addition of the unlabeled lipids. Table 2 indicates that the dilution factors calculated on the basis of the specific activities found are very close to the theoretical values, thus indicating again that ethanolamine plasmalogen is really formed by exchange from the free ethanolamine.

Results showing the incorporation of serine into microsomal lipid by the base-exchange reaction are illustrated in table 3. It is seen that an exchange similar to that described for ethanolamine takes place, although on a quantitative basis the incorporation of serine is not as great as that of ethanolamine, confirming previous results [2]. The apparent discrepancy between the higher incorporation of serine into plasmalogen in experiment (*a*) versus (*c*) and (*d*) is explained in the same way as in the ethanolamine experiments. It must be pointed out, in relation to the experiments of serine incorporation, that no spot

Table 2
Dilution effect on EPG synthesis produced by base-exchange reaction.

Expt. No.	Phospholipid synthesized	Activity*	Specific activity**	Dilution factor***	
				Theoretical	Experimental
1	Diacyl-GPE	15.08	42.0		
	Diacyl-GPE†	15.08	7.27	5.97	5.75
2	Alkenylacyl-GPE	1.50	4.10		
	Alkenylacyl-GPE††	1.50	0.91	4.65	4.50

Brain microsomes (about 2.0 mg protein) were incubated as reported in table 1 for 30 min in presence of 2.5 mM Ca^{2+} (final concentration). Activity was estimated by method (*a*).

* Activity = nmole phospholipid produced × mg protein $^{-1}$ × hr^{-1}.

** Specific activity = nCi incorporated per nmole phospholipid P × 10^3.

*** Theoretical value = nmoles phospholipid P added + membrane phospholipid P/membrane phospholipid P. Experimental value = specific activity undiluted experiment/specific activity diluted experiment.

† 1790 nmoles of diacyl-GPE added after incubation (dilution experiment).

†† 1330 nmoles of alkenylacyl-GPE added after incubation (dilution experiment).

of original alkenylacyl-GPS was found on chromatograms, prior to incubation of microsomes with radioactive serine, thus confirming the almost complete absence of this lipid from nervous structures [5, 16–19] and indicating that a great part of the alkenylacyl-GPE found after incubation and determined by methods (*a*), (*c*) and (*d*), must have been formed by serine exchange with preformed lipid, probably alkenylacyl-GPE.

Tables 1 and 3 indicate that ethanolamine and serine can be incorporated by the calcium-stimulated exchange mechanism also into alkali- and acid-stable lipids. These products have been identified, on the basis of several criteria, as alkylacyl-GPE or -GPS, although a rigid isolation procedure has not been worked out. A radioactivity content was in fact clearly evident in the organic phase on resolving by saponification and solvent partition [12, 13] the acid-stable EPG and SPG. Secondly, results similar to the above were obtained by carrying out the same procedure on the column eluates rather than directly on the lipid extract obtained after incubation. Moreover, a radioactivity content was always evident in the last lower phase lipids obtained after the selective hydrolysis procedures (methods *c* and *d*), even on prolonging up to 60 min the original periods of alkaline and acid hydrolysis [9]. Finally, the EPG- or SPG-containing fractions from the column eluates obtained by methods (*c*) and (*d*) never contained alkali- and acid-stable lipids, other than the alkylacyl-derivatives of EPG and SPG.

Therefore labelled alkylacyl-GPE and -GPS are likely to be produced in rat-brain microsomes by exchange reaction. This is particularly interesting, owing to the low levels in nervous tissue of alkylacyl-GPE [14–16, 19] and to the virtually complete absence of alkylacyl-GPS from the same tissue [19]. Probably, the greater part of the labeled alkylacyl-GPS found in our experiments must have been form-

Table 3
Incorporation of L-[3-^{14}C]serine into SPG of rat-brain microsomes by base-exchange reaction.

Experiments (methods of assay adopted)*	Incorporation levels**		
	Diacyl-GPS	Alkenylacyl-GPS	Alkylacyl-GPS
(*a*)	5.70 (85.6)	0.60 (9.1)	0.35 (5.3)
(*c*)	43.4 (96.2)	1.1 (2.5)	0.58 (1.3)
	30.0 (97.0) 26.9†	0.47 (1.6) 0.40†	0.43 (1.4)
(*d*)	37.2 (97.0)	0.53 (1.4)	0.61 (1.6)

1.5 mM-L-[3-^{14}C]serine (specific activity of 0.493 μCi/μmole) incubated as reported for table 1, in place of ethanolamine. For other explanations of the table, see table 1.

ed by serine exchange with preformed alkylacyl-GPE.

The properties of the enzymic system which produces labeled alkenylacyl- and alkylacyl-lipids by base-exchange have been studied, and were found to be strictly comparable to those reported for the synthesis of the diacyl-lipids [1–3]. Thus, both systems have similar subcellular distribution pattern, similar pH optimum, absolute requirement for Ca^{2+}, and similar Ca^{2+}-concentration-activity relationships. Moreover, when serine acts as the exchanging base only SPG are produced, thus indicating that no decarboxylation on SPG occurs. Whether this result means that the exchange takes place only between bases of similar structure, i.e. between ethanolamine and endogenous EPG or serine and endogenous SPG, is not known, although previous results [2] and the actual findings on the synthesis of alkenylacyl- and alkylacyl-GPS would raise the possibility of exchange also between different nitrogenous bases.

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