# SOLUBILIZATION AND PROPERTIES OF A MEMBRANE-BOUND ENZYME FROM RAT BRAIN CATALYZING A BASE-EXCHANGE REACTION

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SUMMARY

A solubilized base-exchange enzyme activity was dependent upon the addition of phospholipids, added $Ca^{++}$ ion, and had optimum pH of 7.2. Phosphatidylethanolamine was found to be the best stimulator of both $(^{14}C)$-ethanolamine and $(^{14}C)$-serine incorporation. Preliminary evidence suggests the presence of phospholipase D type activity in this solubilized preparation.

A non-energy-dependent, calcium-stimulated incorporation of ethanolamine, L-serine and choline into phospholipids by a particulate preparation from a variety of tissues from different species (1-14), including mammalian nervous system (15-19) has been documented. Phosphatidylserine biosynthesis in mammalian tissues appears to occur solely by this mechanism (2). It has been suggested that this reaction might have a physiological role in the transport of ethanolamine, serine and choline across the surface membrane into the cell (8,16,17).

The transferase activity, reversal of phospholipase D, or a different specific enzyme system has been suggested as the possible mechanism for this base exchange reaction (8,16). Phospholipase D in the higher plants has been shown to catalyze a transphosphatidylation reaction as a reversal of the normal hydrolytic activity (20-22). However, this enzyme has been reported to be absent from mammalian tissues (23).

The present communication documents (1) the solubilization of the enzyme catalyzing the base-exchange reaction from brain particles, (2) the absolute

requirement for exogenous phospholipid acceptors, and (3) the possible presence of phospholipase D activity in the solubilized preparation.

## MATERIALS AND METHODS

Chemicals: The source of radioactive materials was as follows: [1,2-$^{14}$C]-ethanolamine (spec. act. 78 mCi/mmole), [UL-$^{14}$C]-L-serine (spec. act. 120 mCi/mmole), [1,2-$^{14}$C]-phosphatidylcholine chloride (spec. act. 117 mCi/mmole) and [1,2-$^{14}$C]-phosphatidylethanolamine (spec. act. 60 mCi/mmole) from International Chemical & Nuclear Corp., Irvine, Calif.; [methyl-$^{14}$C]-choline chloride (spec. act. 60 mCi/mmole) from Amersham Searle, Chicago, Ill. The mixed soya phospholipids, Asolectin and Inosithin were obtained from Associated Concentrates, New York. Individual phospholipids were purchased from Supelco, Inc., Bellefonte, Pa. and Sigma, St. Louis, Mo. or prepared in this laboratory. The purity of all the lipids was established by thin layer chromatography prior to their use. An amphoteric surface active agent, Miranol H2M, was a gift from the Miranol Chemical Company, Irvington, N.J. Phospholipase D was either from C.F. Boegringer & Soehne GnbH, Mannheim or Sigma Chemical Co.

Solubilization of the enzyme catalyzing the base-exchange reaction: The particles prepared as previously described (16) were suspended in a solution of 1% Miranol H2M in 10 mM HEPES buffer containing 1 mM EDTA, pH 7.2, and sonicated for two 15-second periods at 0°C. The suspension was centrifuged at 166,500 x g for 60 min and the supernatant (15 ml) immediately applied on a column (2.5 x 54 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) which had been equilibrated with the same buffer. The enzyme preparation used throughout these studies was eluted at the void volume, almost completely detergent-free.

Preparation of phospholipids microdispersion: Microdispersions of the mixed soya bean phospholipids, Asolectin and Inosithin, and individual purified phospholipids were prepared essentially according to the method originally reported by Fleischer et al. (24). The mixture was dialyzed at 4°C against approximately 100 volumes of 20 mM HEPES-1 mM EDTA buffer (pH 8.0). The ratio of the optical density values at 500 mμ and 700 mμ and the total phosphorus content were assayed prior to using as substrates.

Enzyme assay of the base-exchange reaction: A typical reaction mixture contained: 10 μmoles of HEPES buffer at pH 7.2 or 7.5; 50 μg of bovine serum albumin; 2 μmoles of $CaCl_2$; phospholipid microdispersion generally equivalent to about 3.0 μg inorganic phosphorus; either 6.4 nmoles (0.5 μCi) of [1,2-$^{14}$C] ethanolamine; or 8.7 nmoles (1.25 μCi) of L-[U-$^{14}$C] serine; or 31.9 nmoles (1.95 μCi) of [methyl-$^{14}$C] choline; and 30 to 50 μg of enzyme protein in a total volume of 0.24 ml, the incubation was at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of ice-cold 10% TCA, the contents of the tubes filtered directly on a 0.45 μ nitrocellulose filter (Millipore Corp., Bedford, Mass.) (26) and the filter was washed with 20 ml of ice-cold 5% TCA. The filter was then dissolved in Aquasol (New England Nuclear, Boston, Mass.)

and assayed for radioactivity in a Packard Model 3380 scintillation spectrometer. The lipid extraction procedure of Folch et al. (27) was also employed for assaying the incorporation of radioactive precursors into individual lipids as previously reported (16). Both methods gave almost identical quantitative results.

Assay of phospholipase D activity in the solubilized enzyme preparation: The basic assay mixture consisted of: 10 μmoles of HEPES buffer, pH 7.5;

4 or 10 μmoles of $CaCl_2$; either (1,2-$^{14}$C)-phosphatidylcholine, 1.4 x $10^5$ total cpm (0.60 nmoles); or (1,2-$^{14}$C)-phosphatidylethanolamine, 7.1 x $10^4$ total cpm (0.59 nmoles) and enzyme (50 to 100 μg protein) in a final volume of 0.2 ml. The samples were incubated, generally for 10 min, at 37°C with shaking and the reaction was terminated by the addition of 0.05 ml (500 μg) of BSA and 0.25 ml of ice-cold 10% TCA. The mixture remained at 0°C for 30 min and then centrifuged at 3,000 rpm for 10 min. Radioactivity present in 100 μl-aliquot of the supernatant was determined in a Packard Model 3380 scintillation counter with Aquasol. The remainder of the supernatant was washed 5 times with 2 volumes of ether and used for product identification.

Identification of reaction product: The lipid products obtained under the conditions of base incorporation from 5 incubations were separated by TLC, as reported previously (16). The radioactive spots were located both with a Berthold thin layer radioscanner (Varian) and by radioautography and identified by cochromatography with authentic phospholipid standards.

The acid-soluble product obtained from the phospholipase D incubations was subjected to paper chromatography with n-butanol-acetic acid-water (4:1:5, v/v) and propanol-acetic acid-water (8:1:1, v/v) as solvents. Phosphorylcholine, glycerylphosphorylcholine, phosphorylethanolamine, (methyl-$^{14}$C)-choline and (1,2-$^{14}$C)-ethanolamine were applied on the same paper as standards. The phosphorus containing materials were detected with Hanes-Isherwood's reagent, followed by the exposure to UV-light, and the radioactive spots were located with a Berthold radioscanner.

Analytical methods: Protein was determined by the method of Lowry et al. (28) with bovine serum albumin as standard and also by measuring ultraviolet spectrum (29). Phospholipids were assayed by determining phosphorus after digestion with perchloric acid (30).

## RESULTS

The reactivation of ($^{14}$C)-ethanolamine incorporation by exogenous phospholipid with the solubilized enzyme is shown in Fig. 1. At low concentrations, both Inosithin and Asolectin were equivalent. Phosphatidylethanolamine isolated from Inosithin gave maximum reactivation at approximately 1 μg phosphorus equivalent, while pure phosphatidalethanolamine gave maximum reactivation at approximately 2.5 μg phosphorus equivalent. The reactivation achieved with Asolectin and Inosithin on the incorporation of ($^{14}$C)-serine into phospholipids was almost identical with that obtained with ($^{14}$C)-ethanolamine. ($^{14}$C)-Choline incorporation into phospholipids was quantitatively less, however, reactivation of the incorporation by exogenous Inosithin and Asolectin was also observed (Table I).

The solubilized preparations exhibited a pH optimum around 7.2 with all three bases as substrate and showed an optimal calcium concentration at 8.3 mM. Detergents were strongly inhibitory for the solubilized enzyme even though

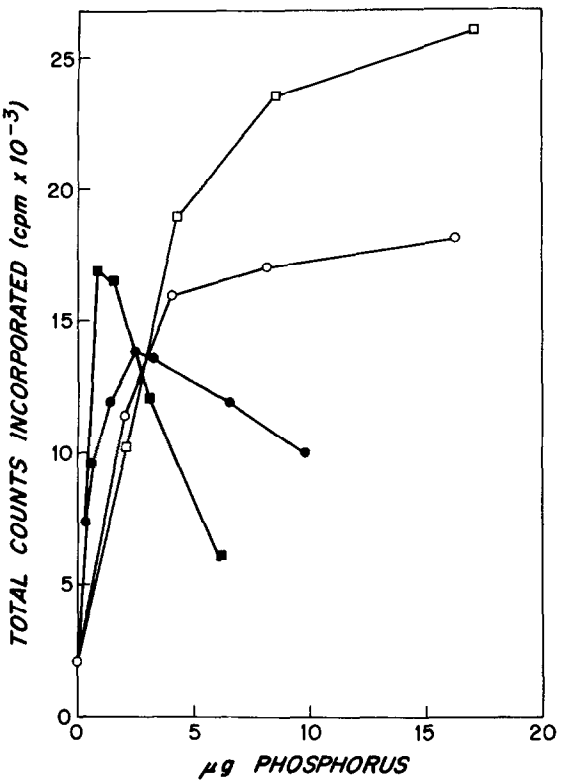


Fig. 1: Reactivation of [1,2-$^{14}$C]-ethanolamine incorporation with the solubilized enzyme by exogenous phospholipids. Various amounts of Inosithin (O), Asolectin (□), phosphatidalethanolamine (●) and phosphatidylethanolamine fraction of Inosithin (■) were added to the incubation mixture. Details are provided in the text.

some were found to be stimulatory for the particulate-bound enzyme.

Phospholipid specificity for the stimulation of base incorporation by the solubilized enzyme was examined, and results are presented in Table I. The ethanolamine phosphoglycerides (EPG) including phosphatidalethanolamine (EPG plasmalogen) exhibited good stimulatory activity for [$^{14}$C]-ethanolamine incorporation into phospholipids, except for distearoyl EPG which showed no activity. This is presumably due to the difficulty encountered in preparation of microdispersion of distearoyl EPG. Microdispersions of pure EPG and phosphatidic acid were found difficult to prepare in water, as noted by several authors (25,31). The other phospholipids caused no stimulation of [$^{14}$C]-ethanolamine incorporation, and cardiolipin seemed to inhibit this incorporation.

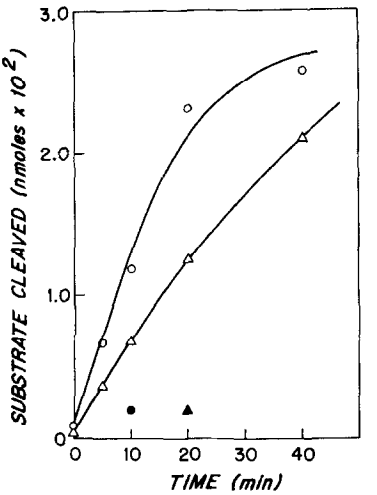


Fig. 2: Release of TCA soluble radioactivity from either base-labelled CPG (in the presence of $Ca^{+2}$ (○), in the absence of $Ca^{+2}$ (●) ) or EPG (in the presence of $Ca^{+2}$ (△), in the absence of $Ca^{+2}$ (▲) ) as a function of time. Details are provided in the text.

The stimulatory activity of EPG for $\{^{14}C\}$-serine incorporation was similar to that observed for $\{^{14}C\}$-ethanolamine. Choline phosphoglycerides (CPG) were only slightly effective in $\{^{14}C\}$-serine incorporation, while serine phosphoglycerides (SPG) were found to be almost ineffective. In the case of $\{^{14}C\}$-choline incorporation, EPG, SPG and CPG showed slight activities.

The products were identified by TLC, followed by radioautography as follows: with $\{^{14}C\}$-ethanolamine, the products cochromatographed with phosphatidylethanolamine and lysophosphatidylethanolamine standards, with $\{^{14}C\}$-L-serine, the products cochromatographed with phosphatidylserine standard, with $\{^{14}C\}$-choline, the products cochromatographed with phosphatidylcholine and lysophosphatidylcholine standards.

Phospholipase D activity as a function of incubation time with base-labelled pure $\{^{14}C\}$-CPG and $\{^{14}C\}$-EPG is presented in Fig. 2. These substrates were found to be cleaved by commercial preparations of plant phospholipase D (32). Radioactive choline was detected in the incubation with $\{^{14}C\}$-CPG, while radioactive ethanolamine was found with $\{^{14}C\}$-EPG as detected by paper chromatography (Table I). In addition, radioactive phosphatidic acid was produced in incubations with $(U\text{-}L\text{-}^{14}C)$-CPG.

TABLE 1: Phospholipid specificity for reactivation of the solubilized enzyme in incorporation of $^{14}$C-ethanolamine, $^{14}$C-serine and $^{14}$C-choline.

| Phospholipid added | | Ethanolamine Incorporated | Serine Incorporated | Choline Incorporated |
|---|---|---|---|---|
| | µg-phosphorus/incubation | TOTAL COUNTS INCORPORATED (cpm)* | | |
| None | | 1710 | 2537 | 1894 |
| Asolectin | 3.91 | 19743 | 17364 | 4747 |
| Inosithin | 3.55 | 16650 | 12670 | 4147 |
| EPG fr. of Inosithin | 1.53 | 11322 | 8792 | 4849 |
| EPG Folch fr. of egg | 1.00 | 7683 | | |
| EPG bovine | 0.58 | 6224 | 5150 | 3600 |
| Dioleoyl EPG | 0.60 | 6111 | 3270 | 3407 |
| Distearoyl EPG | 0.12 | 1690 | 2450 | 1826 |
| EPG plasmalogen | 0.65 | 10029 | 6069 | 3686 |
| CPG fr. of Inosithin | 1.93 | 1399 | | 2074 |
| CPG (1) | 2.66 | 1704 | 4636 | 3589 |
| CPG (2) | 2.44 | 1793 | 3108 | 2770 |
| SPG (1) | 1.72 | 1242 | 2550 | 4001 |
| SPG (2) | 1.60 | 850 | 2479 | 3815 |
| PA (1) | 0.69 | 1627 | | |
| PA (2) | 0.52 | 1446 | | |
| IPG | 1.38 | 1410 | | |
| GPG | 0.54 | 1455 | | |
| Cardiolipin | 2.12 | 0 | | |

Each incubation contained 42.9 µg-protein of the solubilized enzyme and the individual phospholipid. Details of the incubation and assay system are provided in the text. Abbreviations used: EPG, ethanolamine phosphoglyceride; CPG, choline phosphoglyceride; SPG, serine phosphoglyceride; PA, phosphatidic acid; IPG, Inositol phosphoglyceride; GPG, glycerol phosphoglyceride.

* The boiled enzyme values of approximately 800 cpm have been subtracted.

## DISCUSSION

Hübscher (4) suggested that the base-exchange reaction observed in tissues might be the reversal of phospholipase D activity while Porcellati et al. (8) suggested that the reaction is due to a different single enzyme system.

EPG's were the most stimulatory for both ethanolamine and serine incorporation,

TABLE 2: Identification of $^{14}$C-choline and $^{14}$C-ethanolamine as products of phospholipase D activity of the solubilized enzyme by paper chromatography.

| | Butanol-Acetic acid-Water (4:1:5) | Propanol-Acetic acid-Water (8:1:1) |
|---|---|---|
| | $R_f$ | $R_f$ |
| Product (1) | 0.454 | 0.229 |
| Choline | 0.438 | 0.219 |
| Phosphorylcholine | 0.068 | 0.040 |
| Glycerylphosphorylcholine | | 0.047 |
| Product (2) | 0.650 | 0.358 |
| Ethanolamine | 0.642 | 0.371 |
| Phosphorylethanolamine | 0.049 | 0.026 |

Product (1) : obtained when (1,2-$^{14}$C)-phosphatidylcholine microdispersion was used as substrate
Product (2) : obtained when (1,2-$^{14}$C)-phosphatidylethanolamine microdispersion was used as substrate

compared to the other phospholipids tested, while SPG's were almost inactive. These results do not support the suggestion by Borkenhagen et al. (2) and Hübscher et al. (1,4) that ethanolamine is exchanged for the serine moiety of SPG.

The possible presence of phospholipase D activity in the solubilized preparation was suggested from studies using base-labelled CPG and EPG as substrates. The release of acid-soluble radioactivity was found to be a function of both time and calcium concentration; both choline and ethanolamine were detected as products as judged by paper chromatography. The cleavage of EPG by the solubilized enzyme is slower than that of CPG, as reported with cabbage phospholipase D by Dawson et al. (32). It is suggested that the enzyme catalyzing the base-exchange reaction in mammalian nervous tissues might be similar to that of phospholipase D in higher plants.

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