C-6 or shorter chains do not form extended bilayers in aqueous solution even at concentrations above their critical micelle concentrations but instead form small micelles (4). The properties of lyso-PC also do not favor aggregates of this phospholipid in mixed micelles with detergent.

In contrast to the stimulatory action of zwitterionic phospholipids, enzyme activity was inhibited by negative phospholipids even in the presence of optimal PC. As shown in Fig. 1, activity in the presence of three different concentrations of PC was determined at increasing concentrations of phosphatidylinositol (PI). 50% inhibition of the enzyme occurrred at PI:PC ratios of 1.0, 1.02, and 0.93, respectively, in the presence of 30 μg, 60 μg, and 90 μg of PC; almost 90% inhibition of activity was observed at a PI:PC ratio of 2.0 in all three cases.

In summary, α-mannosidase appears to require a hydrophobic surface containing closely packed zwitterionic head groups, and the dispersion of these charge groups with either detergent or by dilution with negatively charged groups leads to a decrease in activity. Although the levels of PI which cause significant inhibition of the α-mannosidase in vitro are considerably higher than the amount of PI present in the membranes of golgi or endoplasmic reticulum, preliminary studies indicate that the enzyme is much more sensitive to PI in the absence of Triton X-100. Thus fluctuations in the phospholipid microenvironment of the α-mannosidase could markedly influence the activity of the enzyme.

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RECONSTITUTION OF THE MEMBRANE-BOUND FORM OF DOPAMINE-β-HYDROXYLASE

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In the adrenal medulla and extradrenal chromaffin tissue, chromaffin granules are involved with the biosynthesis, storage, and secretion of catecholamines. Dopamine-β-hydroxylase (DBH), which converts dopamine to norepinephrine, exists as both a soluble and membrane-bound enzyme in the granule. The two forms of hydroxylase purified by others have appeared identical in physical composition.

We report here the purification of both forms of DBH which appear to be different based on gel filtration chromatography and reconstitution into phospholipid-bilayer membranes. A comparison of the enzymatic activity of reconstituted membranous enzyme with soluble enzyme is also presented.

METHODS

Chromaffin granule membranes were isolated from fresh bovine adrenal medullas (1). Extraction of membranes with octyl glucoside (2%, 30 min) was followed by DEAE-cellulose chromatography (10–100 mM sodium phosphate gradient, pH 6.8, 1% octyl glucoside). The eluted hydroxylase was then chromatographed on Sepharose CL-6B (50 mM HEPES, pH 7.4, 100 mM NaCl). Concentration of fractions was done using an Amicon PM30 membrane (Amicon Corp., Lexington, MA). The soluble form of the enzyme was purified from the granule lysate by DEAE-cellulose chromatography (50 mM sodium phosphate pH 6.8, 200 mM NaCl) and gel filtration as above.

Enzyme assays were as described by Wallace et al. (2) in 0.2 M sodium acetate, pH 5.0 (during purification), or in 50 mM MES, pH 6.0 (kinetic experiments). The reconstitution procedure is described in the legend to Fig. 1. All reagents were obtained from Sigma Chemical Co., St. Louis, MO, except ^{14}C-phosphatidyl choline from New England Nuclear, Boston, MA.

RESULTS

The data from a typical purification of bovine adrenal DBH are shown in Table I. Activity of the enzyme is dependent on associated phospholipid and therefore the specific activities shown are only estimates. Four to five molar equivalents of phospholipid are associated with the final fraction of purified hydroxylase as determined by phosphate analysis and semiquantitative thin-layer chromatography.

Hydroxylase purified from the soluble contents of the granules was compared to the purified membranous hydroxylase on sodium dodecylsulfate polyacrylamide gel electrophoresis. Under reducing conditions both forms

TABLE I
PURIFICATION OF MEMBRANOUS DOPAMINE-β-HYDROXYLASE

Fraction	Total protein (mg)*	Activity‡	Yield§
Octyl Glucoside Extract	59	0.58	100
DEAE-Cellulose	6	1.60	28
Sepharose-CL-6B	4	3.46	40

*Starting material was 70 mg of granule membrane protein.
‡Expressed as μmol octopamine/min/mg protein.
§Yield of enzyme activity as percent of octyl glucoside extract.

showed a major band of apparent molecular weight of 75,000 and a minor band at 70,000 mol wt. This heterogeneity was also observed in an identical pattern from soluble DBH purified by Concanavalin A-Sepharose chromatography as described by Rush et al. (3).

A large difference in Stokes radius was observed between the two forms when analyzed by gel filtration. From the elution positions on a calibrated Sepharose CL-6B column the molecular weight of the soluble form of DBH was estimated to be 300,000 and that of the membranous form, $>10^6$.

Incubation of the purified membranous form of DBH with small unilamellar vesicles (SUV) resulted in binding of most of the enzyme to the vesicles (Fig. 1). Only the lipid-bound enzyme floats to the top of the sucrose. Without lipid neither the membranous nor soluble form of DBH floats and no binding of the soluble form results from incubation with SUV even if the soluble form has been treated previously with detergent.

The effects of increasing substrate concentration on the velocity of enzyme activity for purified soluble and purified, reconstituted membranous DBH were compared. With both forms of the enzyme, nonhyperbolic kinetics were observed. Similar kinetic curves were obtained with the nonreconstituted membranous enzyme. However, the reconstituted membranous enzyme showed a threefold decrease in activity at saturating substrate concentrations. An interpretation of this effect of a membrane environment on the enzyme activity will await more precise data obtained with a variety of phospholipid membranes and assay conditions.

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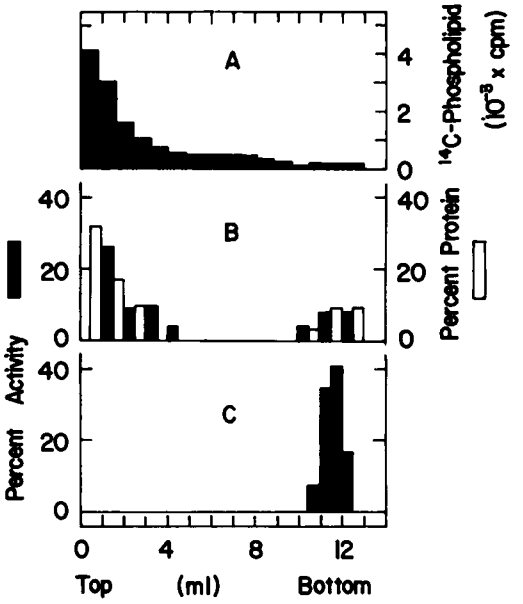

FIGURE 1 Reconstituion of membranous dopamine-β-hydroxylase onto phospholipid vesicles. Sonicated egg lecithin vesicles (2 μmol in 50 mM HEPES, pH 7.4, 100 mM NaCl) were sized as described (4) and mixed with 2.4 M sucrose alone or with sucrose plus 60 μg membranous hydroxylase and brought to 1.2 M sucrose, final. The mixture was incubated for 10 min at 4°C and overlaid with 10 ml of 1 M sucrose and 1 ml of 50 mM HEPES, pH 7.4, 100 mM NaCl. The gradient was centriguated at 130,000 *g* for 16 h and fractionated. The amount of enzyme in each fraction is expressed as percent of total in all fractions. *A.* Sonicated vesicles alone. An identical distribution of lipid was obtained in experiment. *B.* Vesicles plus membranous hydroxylase. *C.* Membranous hydroxylase treated as in *B* with no vesicles added.

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