The Binding of Noradrenaline to Phosphatidylserine. *In Vitro* Studies of an Artificial Model System of Biological Origin

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SUMMARY

Phosphatidylserine chromatographically purified from human hypothalamus shows a high capacity to bind nonionized noradrenaline *in vitro*. Experimental results from a two-phase model, consisting of an aqueous phase with noradrenaline and a lipophilic organic phase containing the purified lipid, are found to agree with theoretical predictions on the effects of pH and concentration on binding capacity. The results make it possible to suggest a mechanism for the transport of noradrenaline through the membranes of the brain.

INTRODUCTION

The rates at which organic molecules penetrate biological membranes are in the range that would be expected for a process of facilitated diffusion through a bimolecular lipid leaflet. This passage of solutes through the membranes is also dependent on the membrane protein (1, 2).

The central nervous system (CNS) membranes contain both glycolipids and phospholipids; the amounts of either may change under different biological conditions (3, 4).

In the present work phosphatidylserine (PS) is shown specifically to bind noradrenaline (NA). This phospholipid is a constituent of all membranes, including those of synaptosomes and synaptic vesicles (5). PS shows a capacity for binding uncharged NA in a simplified two-phase model. The partition of NA is described as a function of pH and concentration.

MATERIALS AND METHODS

Chemicals of highest commercially available purity from British Drug Houses were used for all studies. L-Noradrenaline was obtained from Hoechst Ltd., Germany. This catecholamine was dissolved in deionized water in concentrations of 25 mg and 50 mg per liter. Solutions were stable at least 14 days when kept in well closed bottles.

In studies of the dependence on pH of the binding of NA to PS, three buffer systems were used. These were 0.1 M phosphate buffer in the pH range 5.00–8.00, 0.1 M glycine in 0.1 N NaCl with 0.1 N HCl from pH 2.00 to 3.60 and 0.1 M glycine in 0.1 N NaCl with 0.1 N NaOH from pH 8.40 to 13.00.

Brain material. Four human brains were used for isolation of the lipids. All brains were obtained from individuals who had not suffered from organic neurologic diseases.

Extraction of tissue. The hypothalami of brains were removed and frozen (—20°) within 12 hr postmortem. In each procedure about 4.0 g of hypothalamus was washed in 0.9% saline to remove any blood and then homogenized in a Potter-Elvehjem homogenizer with 25 volumes of chloroform–methanol (2:1 v/v). Extraction was continued by allowing the mixture to stand in an atmosphere of N_2 for 16 hr at 20°. The extracts were filtered through prewashed filter paper. The residue was washed twice with the solvent mixture. The solvent

was removed from the combined extracts at 50° under vacuum. The pressure was increased to 1 atm with N_2. The total chloroform–methanol extractable matter was determined gravimetrically. The lipid material was redissolved in chloroform for column chromatography.

Thin-layer chromatography (TLC). The purity of the lipids was determined by ascending thin-layer chromatography (6). An alkaline chloroform–methanol–25% NH_3 mixture (70:30:5 v/v) was used as the solvent system. The spots were identified by spraying the developed chromatograms with (a) ammonium heptamolybdate in 0.1 N HCl, 70% $HClO_4$, (b) ninhydrin in acetone, (c) 2% resorcinol in 0.1 M $CuSO_4$ in conc. HCl (3) followed by drying and heating to 100°.

Column chromatography. PS was isolated by chromatography on acetylated diethylaminoethyl (DEAE)-cellulose and silicic acid (7–9). About 200 mg of the hypothalamic lipid extract was dissolved in chloroform and was applied to a 2.5 × 25 cm DEAE-cellulose column. Discontinuous elution with mixed lipid solvents gave rise to the following fractions: Chloroform–methanol (8:1), cholesterol + ceramide + cerebroside + lecithin + sphingomyelin; chloroform–methanol (3:2), phosphatidylethanolamine. The methanol elution gave water-soluble nonlipid salts + amino acids + sugars. Glacial acetic acid eluted free fatty acids + PS + gangliosides. The composition of the fractions was visualized by TLC as described above. PS was isolated from the glacial acetic acid fraction by chromatography on silicic acid (9).

No detectable hydrolysis of PS occurred in glacial acetic acid as judged by TLC. The column method used for isolation of PS from the glacial acetic acid fraction was as follows: The lipids extracted from the dried glacial acetic acid fraction were applied to the column. Chloroform eluted free fatty acids, chloroform–methanol (9:1) eluted an unidentified lipid, chloroform–methanol (4:1) eluted PS, and methanol eluted gangliosides. The PS fraction revealed only one spot by TLC by staining with ammonium heptamolybdate or ninhydrin, as described. Resorcinol did not reveal any carbohydrate components. The presence of contaminating phosphoinositides in the purified PS would have been detectable by TLC, since they did not show any migration in the solvent used (R_F for PS = 0.06).

Infrared analysis. PS was isolated by evaporation on a KBr disk made from 100 mg KBr in a Unicam evacuable die mill (MKIII) under 25 tons pressure and 15 mm Hg vacuum. The PS fraction applied to the KBr disk (1 mg/100 mg KBr) was analyzed in a Unicam SP-200 infrared spectrophotometer using a 100-mg KBr disk as reference. The spectrum obtained was analyzed using the law of group frequencies (10). The results confirmed that the material isolated was PS.

Determination of NA. NA was fluorimetrically determined by a slightly modified trihydroxyindole method (11). In this method, NA is oxidized at pH 6.50 by potassium hexacyanoferrate(III). Oxidation was stopped with sodium sulfite, and rearrangement to norlutin was performed by the immediate addition of 10 N NaOH. The fluorescence of norlutin was stabilized by the addition of mercaptoethanol, which in these experiments was found to be the most suitable antioxidant. The reaction mixture consisted of 400 μl of deionized water, 200 μl 0.1 M phosphate buffer pH 6.50, 20 μl 2.5×10^{-3} % $CuCl_2$, 100 μl 0.25% $K_3Fe(CN)_6$ and 100 μl NA solution. After 3 min the oxidation was stopped with 100 μl 33% $Na_2SO_3 \cdot 7\ H_2O$, 10 μl mercaptoethanol and 200 μl 10 N NaOH. After 10 min, samples and blanks were read in a Photovolt fluorometer at 405/520 mμ (activating/emitting wavelengths). The NA content in each sample was calculated by interpolation from a standard curve (concentration interval 0.25–2.75 μg NA) checked in each experiment.

Binding assay. The cell-free system used consisted of centrifuge tubes containing as organic phase (phase II) 400 μl chloroform–methanol (2:1 v/v) with purified PS (1 mg/ml) and 400 μl aqueous phase (phase I) with NA and buffer. A total amount of 10 μg of NA was used in all experiments.

In each run two tubes without lipid were used as controls, while two others containing lipid served as the experimental system. Each mixture was shaken 1 min in N_2 atmosphere to avoid oxidation of PS and NA; after 5 min to clear the phases, these were further separated by centrifugation in a Sorvall RC-2 superspeed centrifuge at 10,000 *g* for 5 min. Two aliquot portions from phase I were removed from each tube for NA determination. All values were thus measured four times with a standard deviation of ±4.4%.

Bound NA could be liberated by shaking phase II with 0.01 N HCl. During initial experiments the extraction yield was found to be 86–90%. By the use of TLC it was found that all the lipid remained in phase II.

RESULTS

Specificity

There was no binding of NA to sphingomyelin, lecithin (12), phosphatidylethanolamine, cerebroside, or sialic acid-free glycolipids. Each lipid was studied in the two-phase system described.

Effect of pH

In the two-phase model used here with PS as the lipid in the nonpolar phase II and NA in the aqueous phase I, there occur the following equilibria:

$$NA_I + H^+ \leftrightarrows NA_IH^+ \quad (1)$$

$$NA_I \leftrightarrows NA_{II} \quad (2)$$

$$NA_{II} + L \leftrightarrows NA_{II}L \quad (3)$$

The corresponding equilibrium constants are

$$K_1 = \frac{[Na_IH^+]}{[H^+][NA_I]}, \quad K_2 = \frac{[NA_{II}]}{[NA_I]}, \quad K_3 = \frac{[NA_{II}L]}{[NA_{II}][L]}$$

where NA_I is the concentration of uncharged NA at a given pH in phase I and NA_IH^+ is the concentration of protonated NA in phase I.

A certain amount of nonionized NA ($=NA_{II}$) is dissolved in phase II, and in this nonpolar state the binding to PS takes place. Equilibrium (3) can be interpreted as a solution of NA_{II} where PS acts as the solvent.

From equilibrium constants one finds

$$[NA_I] = \frac{[NA_IH^+]}{K_1[H^+]} = \frac{[NA_{II}]}{K_2}$$

and

$$[NA_{II}] = \frac{[NA_{II}L]}{K_3[L]} = \frac{[NA_IH^+]K_2}{K_1[H^+]}$$

Thus,

$$[H^+] = \frac{[NA_IH^+]K_3K_2[L]}{[NA_{II}L]K_1} \quad \text{with} \quad \frac{K_3K_2[L]}{K_1} = K_0$$

$$pH = \log \frac{[NA_{II}L]}{[NA_IH^+]} - \log K_0$$

The Partition of NA

This partition into the organic phase is shown in Fig. 1, where the amount of NA_{II} is plotted against pH (13). Fluorometric determinations of NA in phase I before and after reaching equilibrium in the two-phase system make it possible to calculate the partition of NA in phase II both in the presence and absence of PS. On this basis the $NA_{II}L$ concentrations are calculated. Concerning the calculation of NA_IH^+ in the two-phase system containing PS, the concentration of NA in polar phase I is taken to be approximately equal to NA_IH^+. This assumption is supported by the fact that the pK_a-value for the α-amino group in noradrenaline (14) is about 10; thus in Table 1, for example, at pH 10.1, 58.5% NA_IH^+ was found in phase I.

TABLE 1
Partition of noradrenaline in phases I and II[a]

pH	NA_IH^+	NA_{II}	$NA_{II}L$	$\frac{NA_{II}L}{NA_IH^+}$
5.60	98.1	0.0	1.8	0.0183
6.03	90.0	2.5	7.5	0.0833
6.53	82.8	6.0	11.4	0.1376
7.16	81.1	8.0	11.2	0.1400
8.00	74.3	10.0	15.6	0.2099
9.28	71.1	15.0	14.0	0.1969
9.70	65.3	19.1	15.7	0.2404
10.10	58.5	25.3	16.6	0.2837

[a] Data are percents of total NA.

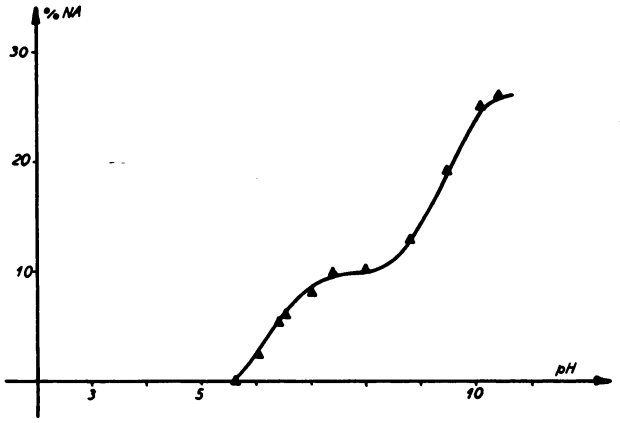

FIG. 1. *The solubility of noradrenaline in pure phase II as function of pH*

The amounts of NA solubilized, i.e., NA_{II}, are expressed as percent of the total NA. Equal volumes of phase I and phase II were shaken; after the phases were cleared, the NA content in phase I was fluorometrically determined.

The Binding of NA_{II} to PS

The binding as a function of pH is shown in Table 1, where consecutive values of NA_IH^+, NA_{II}, and $NA_{II}L$ partitioned in the two phases are expressed as percentage of total NA. The results represented here fit with the equilibrium systems mentioned above. It should be noted that no binding takes place at pH values below 5.60, as NA exists entirely in the form NA_IH^+. However, increasing pH values give increasing NA_{II} and $NA_{II}L$ concentrations, corresponding to an equilibrium shift to the left of equilibrium 1. Figure 2 shows log $(NA_{II}L)/(NA_IH^+) + 2$ plotted against pH.

Effect of NA Concentration

At a given pH value and PS concentration there exists according to the equilibrium $NA_{II} + L \rightleftharpoons NA_{II}L$ a certain maximum number of NA molecules which can be bound. If the equivalent amounts of PS molecules are called n_0 and the amounts of PS molecules bound to NA_{II} at a given total NA concentration are called n, then the Langmuir isotherm $\alpha = c/(c + K)$ applies, where c is the concentration of NA in phase I, K is the equilibrium constant, and α is the degree of saturation (n/n_0).

Figure 3 shows the plot of α against c at pH 7.07. In this experiment the concentra-

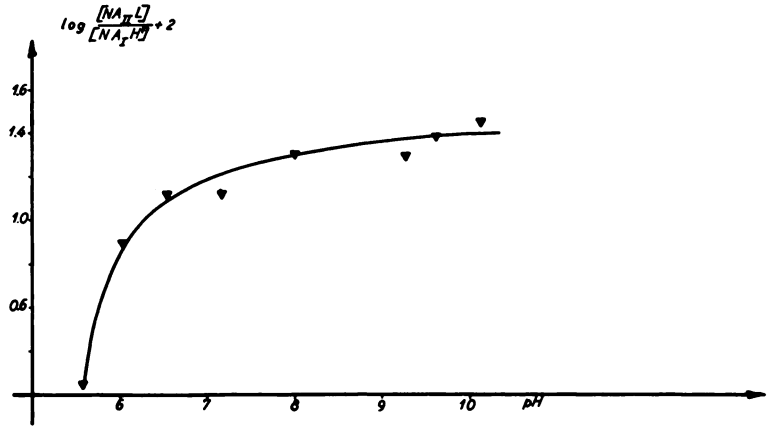

FIG. 2. *log $(NA_{II}L)/(NA_IH^+) + 2$ as function of pH*

In the experiments the concentration of receptor lipid and NA were kept constant.

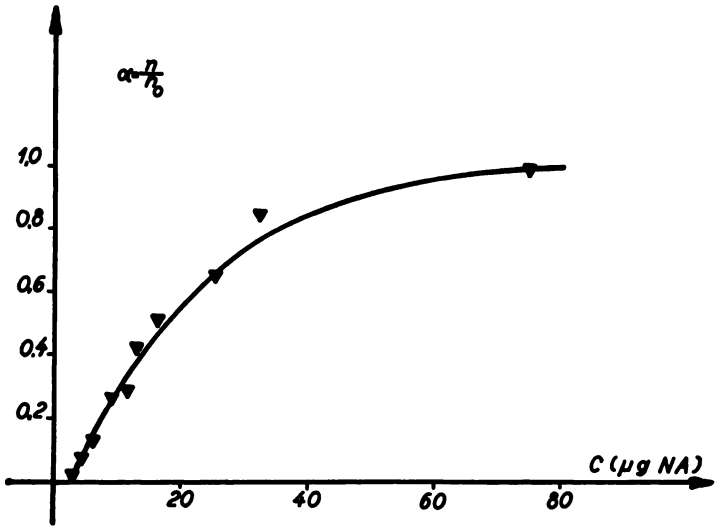

FIG. 3. *Saturation degree* $\alpha = n/n_0$ *against c at pH 7.07*

Here n is equivalent of NA_{II} bound to PS at a given total NA concentration and n_0 the maximum equivalent of NA_{II} which can be bound; c is the concentration of NA in phase I at the given total NA concentration. The volumes of phase I and phase II were equal, and these as well as pH were kept constant.

tions of NA were varied, while volumes of phases I and II were kept constant. The results are in agreement with the above equilibrium, where the activities of all reactants are unity.

$\Delta F°$ *for Formation of NA-PS*

From Fig. 3 is calculated the concentration of NA required to half-saturate PS. This is 20 µg/0.4 ml and indicates a K value of 3380

$$\begin{aligned}\Delta F° &= -RT\ln K \\ &= -1.98 \times 233 \times 2.3 \log 3380 \\ &= -4.67 \text{ kcal}\end{aligned}$$

This value agrees with the possibility of an electrostatic bond between NA and PS (14).

DISCUSSION

Phosphatidylserine, chromatographically purified from human hypothalamic area, occurs in brain membranes in sufficient quantities to explain the specific binding (15). Although the hypothalamic area is a part of the brain with high NA content (16, 17), the PS fraction itself did not contain NA when measured with the fluorometric method. Possible binding of NA to phosphatidylinositol was not studied.

A two-phase model consisting of one polar aqueous phase and one nonpolar organic phase has been used in an attempt to explain how NA in the presence of binding lipid could diffuse through the membrane. The solubility of NA in the lipid area of the membrane is unknown, but in the present system it is sufficient at physiological pH to establish binding with PS, although the pH of lipophilic compartments such as membranes cannot be defined. How NA in the lipophilic milieu reacts with PS is not exactly known, but it might be an acid-base reaction. Since the organic solvent itself is unable to take up protons, the reaction rate for $NA_{II} + L$ must be different from a similar one taking place in aqueous medium. The calculation of $\Delta F°$ for formation of PS-NA reveals the possibility of an electrostatic bond. At physiological pH *in vivo*, the liberation of NA from the diffusible compound $NA_{II}L$ could take place by some kind of ion exchange (18) after diffusion through the membrane

$$\{PS - COO^{-+}H_3N - NA\} + \{ion^+\} \rightarrow \{PS - COO^{-+}ion\} + \{^+H_3N - NA\}$$

So that the NA is liberated as the ion originally taken into the membrane in non-ionized form. The present data may also be related to studies of the binding of other transmitter substances to polar lipids. Thus studies of the binding of serotonin seem to indicate galactolipids or glycolipids as special receptors (19). Results from studies on rat liver plasma membranes (20) indicated that the carboxyl group of a sialic acid conjugate acts as a cation receptor in the active cation transport. However, from the same laboratory it was later shown (21) that no gangliosides could be isolated from the plasma membrane; all the sialic acid was recovered in the protein fraction. According to the present work, the cation receptor could be a sialic acid-free polar lipid such as PS. In summary, information has now been gained supporting the view of a significant role of polar lipids as uptake sites for NA.

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