Reconstitution of 5-Hydroxytryptamine Transport from Cholate-Disrupted Platelet Plasma Membrane Vesicles†

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ABSTRACT: The bile acid cholate disrupts platelet plasma membrane vesicles and inactivates 5-hydroxytryptamine transport activity. The transporter appears not to be solubilized by this procedure, but remains associated with a high molecular weight aggregate. Merely removing cholate does not restore the vesicular structure or transport activity, but in the presence of added soybean phospholipids, cholate removal generates proteoliposomes which accumulate 5-hydroxytryptamine. This reconstituted transport activity is similar in its ionic requirements and inhibitor sensitivity to transport in native membrane vesicles and intact platelets.

Plasma membrane vesicles isolated from blood platelets accumulate 5-hydroxytryptamine (5-HT)[1] against a concentration gradient of up to 500-fold when appropriate ionic gradients are imposed across the vesicle membrane (Rudnick & Nelson, 1978). This process requires external Na^+ and Cl^- and is driven by gradients of Na^+ (out > in) and K^+ (in > out). Transport is inhibited by tricyclic antidepressant drugs such as imipramine, and ionophores such as gramicidin, nigericin, and monensin which dissipate the transmembrane Na^+ and K^+ gradients (Rudnick, 1977). In these respects 5-HT transport into membrane vesicles is identical with 5-HT transport into intact platelets (Sneddon, 1969, 1971; Lingjaerde, 1971; Feinstein et al., 1977).

Transport proteins from a wide variety of membrane sources have been solubilized and reconstituted with lipids to form proteoliposomes which retain transport activity (Kagawa & Racker, 1971; MacLennan, 1970; Shertzer & Racker, 1974; Knowles et al., 1975; Kasahara & Hinkle, 1976; Crane et al., 1976). One widely used technique for reconstitution of transport in liposomes has been developed by Racker and co-workers and involves solubilization of the membrane with the bile acid cholate, followed by removal of cholate in the presence of phospholipids by dialysis (Kagawa & Racker, 1971; MacLennan, 1970; Racker, 1972). In the present study, the disruption of platelet plasma membrane vesicles by cholate and reconstitution of transport activity with phospholipids are described.

Materials and Methods

[1,2-^{3}H]-5-HT was obtained from New England Nuclear. Imipramine was a gift of Mr. Charles Brownley, Geigy Pharmaceuticals; monensin was a gift of Dr. Robert Hosley, Lilly Research Laboratories; nigericin was kindly donated by Dr. Julius Berger, Hoffmann La-Roche, Inc. Cholate was purified from cholic acid (Sigma) by charcoal treatment and recrystallization from 70% ethanol. Crude soybean phospholipids obtained from Associated Concentrates, Woodside, N.Y., were washed as described by Kawaga & Racker (1971) prior to use. All other materials were reagent grade obtained through commercial sources.

Plasma membrane vesicles were prepared from human platelets by the method of Barber & Jamieson (1970) as described previously (Rudnick, 1977). Transport assays were performed as described previously (Rudnick, 1977). Reconstituted proteoliposomes prepared in potassium phosphate buffer were centrifuged for 1 h at 300 000g, resuspended in 0.1 M potassium phosphate, pH 6.7, containing 1 mM $MgSO_4$, and assayed by the same method used for native vesicles. Protein was determined by the method of Lowry et al. (1951). Samples were prepared for electron microscopy by a modification of the procedure of Sabatini et al. (1963). Samples were fixed in 0.05 M sodium cacodylate buffer, pH 7.3, containing 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. The Epon blocks were sliced on a Porter Blum MT-2 ultramicrotome, stained with lead citrate and uranyl acetate, and viewed under a Zeiss 9-52 electron microscope.

Results

As shown in Figure 1, sodium cholate inhibits 5-HT transport by isolated platelet plasma membrane vesicles. Half-maximal inhibition of the steady-state level of 5-HT accumulation is observed at approximately 0.5 mM, and essentially complete inhibition is achieved at cholate concentrations of 10 mM. Although data are not shown, it is noteworthy that, above concentrations of 15–20 mM, cholate inhibition is not reversible by 20-fold dilution into cholate-free medium. This apparently irreversible inhibition by 20 mM cholate is complete over a range of membrane protein concentrations from 1 to 15 mg of membrane protein per mL.

Cholate is essentially completely removed from phospholipid vesicles by gel filtration (Brunner et al., 1976). When platelet plasma membrane vesicles suspended in 25 mM cholate are applied to a Sephadex G-50 column and eluted with cholate-free buffer, a turbid peak elutes at the void volume. This material does not take up 5-HT when diluted into NaCl medium (Figure 2), suggesting that cholate irreversibly denatures either the 5-HT transporter itself, or the ability of the membrane to act as a permeability barrier. When crude soybean phospholipids are added to cholate-denatured membrane vesicles, and the resulting mixture is chromatographed on Sephadex G-50, the void volume peak contains vesicles which regain 5-HT transport activity (Figure 2).

The loss of transport activity in cholate, and its reconstitution by exogenous phospholipids, is mirrored by corresponding

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[1] Abbreviation used: 5-HT, 5-hydroxytryptamine.

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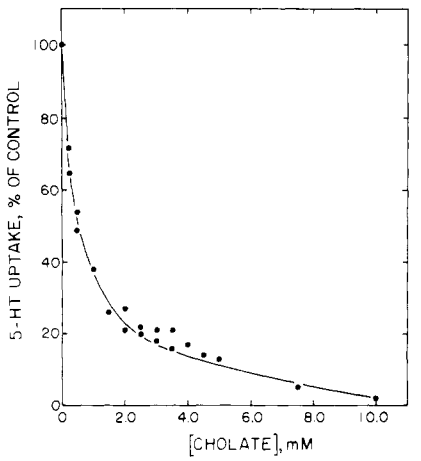

FIGURE 1: Inhibition of 5-HT transport by sodium cholate. A suspension of membrane vesicles (2.9 mg of membrane protein per mL) in 0.1 M potassium phosphate buffer, pH 6.7, containing 1 mM $MgSO_4$ was diluted 20-fold into 0.1 M NaCl containing 0.165 μM [^{3}H]-5-HT, 1 mM $MgSO_4$, and the indicated amount of sodium cholate. Uptake was measured after a 5-min incubation at 25 °C. Control vesicles assayed in the absence of cholate accumulated 119 pmol of 5-HT per mg of membrane protein in 5 min.

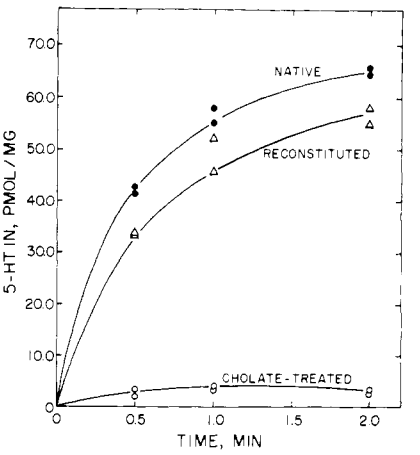

FIGURE 2: Uptake of 5-HT by native membrane vesicles, cholate extracted vesicles, and reconstituted proteoliposomes. A suspension of membrane vesicles (11.3 mg of membrane protein per mL) in 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM $MgSO_4$ was mixed with an equal volume of 50 mM potassium cholate in the same buffer. Alternatively, vesicles were mixed with an equal volume of 50 mM potassium cholate in buffer containing 20 mg of soybean phospholipids per mL. A sample of 0.2–0.5 mL was then applied to a 1 × 30 cm column of Sephadex G-50 (fine) and eluted at room temperature with 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM $MgSO_4$. The turbid peak eluting at the void volume of the column was centrifuged and assayed as described under Materials and Methods. Only Na^+-stimulated uptake is shown. When Li^+ replaced Na^+, uptake was less than 5 pmol per mg of membrane protein. (●—●) Native vesicles; (○—○) cholate-treated vesicles; (△—△) cholate-treated vesicles to which phospholipids were added before gel filtration.

ultrastructural changes, as shown in Figure 3. Panel A shows native membrane vesicles which have been stored at −80 °C, thawed, and washed in potassium phosphate buffer. Addition of 25 mM cholate and gel filtration completely destroy the vesicular nature of the preparation, yielding an amorphous precipitate of protein and lipid (Figure 3B). When exogenous lipids are added prior to cholate removal, however, vesicles are formed (Figure 3C) which appear to be closed structures, although they are smaller than native vesicles and are mixed with granular elements which probably represent denatured protein and lipid. The correlation of vesicular structures with transport suggests that cholate does not irreversibly denature the 5-HT transporter, but that cholate-disrupted plasma membrane components do not reassociate properly when cholate is removed. In the presence of soybean lipids, however, plasma membrane components, including the transporter, associate with phospholipid during cholate removal, yielding vesicles which transport 5-HT. These proteoliposomes contain the buffer in which they were prepared, potassium phosphate, and when diluted into NaCl medium, gradients of Na^+, K^+, and Cl^- are imposed across the vesicle membrane. These gradients are of the same direction and magnitude as have been shown to maximally stimulate 5-HT transport in native membrane vesicles (Rudnick, 1977).

Table I presents additional evidence that transport of 5-HT into reconstituted proteoliposomes is identical with transport into plasma membrane vesicles. 5-HT accumulation requires both Na^+ and Cl^- in the external medium. Moreover, the tricyclic antidepressant imipramine inhibits 5-HT transport in reconstituted vesicles as well as in intact plasma membrane vesicles (Rudnick, 1977). Finally, the ionophores nigericin and monensin inhibit transport in reconstituted vesicles, indicating that transport is driven by the transmembrane gradients of Na^+ and K^+ imposed by dilution of proteoliposomes into NaCl medium. Thus, the procedure of cholate disruption and reconstitution does not alter the functional characteristics of the 5-HT transporter.

Although 25 mM cholate visibly decreases the turbidity of membrane vesicle suspensions, a slight opalescence remains, suggesting that large protein or lipoprotein aggregates remain after the vesicles are disrupted. To determine the extent to

TABLE I: Inhibition of the Initial Rate of 5-HT Transport into Native and Reconstituted Membrane Vesicles.[a]

conditions	inhibition of 5-HT uptake rate (%) native	reconstituted
control	0	0
$[Na^+] = 0$	88.7	90.6
$[Cl^-] = 0$	95.4	98.4
+1 μM imipramine	89.7	84.8
+5 μM nigericin	71.1	76.8
+5 μM monensin	83.4	78.0

[a] Native vesicles and reconstituted vesicles prepared as described in the legend to Figure 2 were assayed for 15 s at 25 °C under the conditions given below. Na^+ was replaced with Li^+ and Cl^- was replaced with phosphate. Imipramine was added to the vesicles at the same time as [^{3}H]-5-HT. Nigericin and monensin were added to the vesicles 5 min prior to assaying transport. Control uptake rate for native vesicles was 120.3 pmol/(mg min) and for reconstituted vesicles was 90.2 pmol/(mg min).

which the 5-HT transporter is associated with these aggregates, cholate-solubilized vesicles were applied to a column of Sepharose 6B and eluted with buffer containing 25 mM cholate. Figure 4 shows a typical elution profile. A visibly turbid peak was eluted in the void volume, followed by a broad peak of soluble material. These peaks were pooled, mixed with soybean phospholipids, and concentrated. Cholate was then removed by gel filtration on Sephadex G-50, forming proteoliposomes which were assayed for transport activity. As shown in Table II, the turbid peak, pool 1, contained most of the activity but only 23% of the protein recovered from the column. Pools 2 and 3, which contained proteins soluble in 25 mM cholate, together accounted for only 26% of the total activity, although they represented most of the protein eluted from the

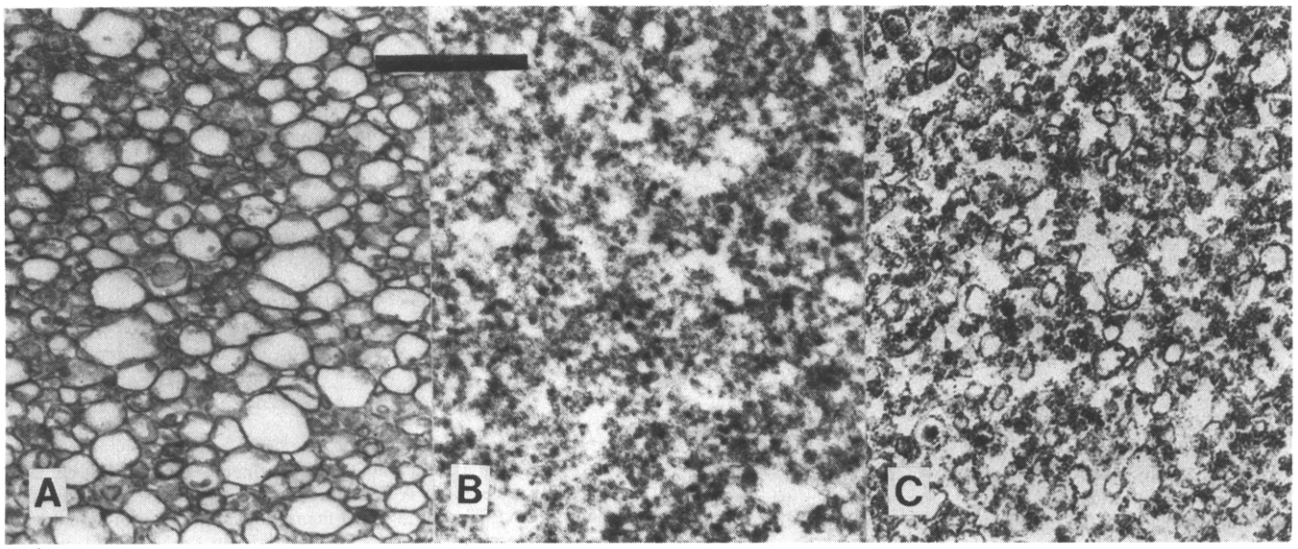

FIGURE 3: Electron microscopy of native, cholate-treated, and reconstituted vesicles. Vesicles prepared as described in the legend to Figure 2 were prepared for electron microscopy as described under Materials and Methods. (A) Native vesicles; (B) cholate-treated vesicles; (C) cholate-treated vesicles reconstituted with soybean phospholipids. The bar is 1 μm. All micrographs are shown in the same magnification.

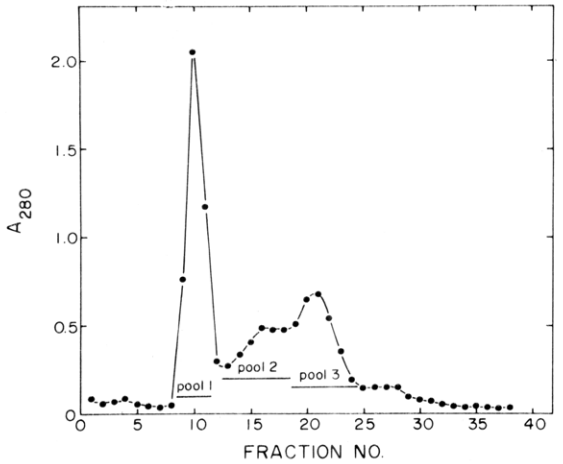

FIGURE 4: Cholate gel filtration of cholate-treated membrane vesicles in the absence of exogenous phospholipids. Membrane vesicles (11.3 mg of membrane protein per mL) suspended in 0.1 M potassium phosphate buffer, pH 8.0, containing 25 mM potassium cholate and 1 mM $MgSO_4$ were applied to a 1 × 30 cm column of Sepharose 6B and eluted with the same buffer at 4 °C. The indicated fractions were pooled, and soybean phospholipids dissolved in 25 mM potassium cholate were added (2 mg of phospholipid per sample of 0.4 to 1.0 mg of protein). The pooled samples with phospholipid were concentrated by dialysis against 20% (w/v) poly(ethylene glycol) (15 000 to 20 000 mol wt) and cholate was removed by gel filtration as described in the legend to Figure 2.

TABLE II: Recovery of Transport Activity and Protein after Cholate–Gel Filtration.[a]

	protein		transport act.	
pool	mg	% of total	pmol per mg of protein	% of total
1	0.388	23	75.8	74
2	0.69	42	6.5	11
3	0.59	35	10.13	15

[a] Membrane vesicles were disrupted with cholate and gel filtered as described in the legend to Figure 4. Proteoliposomes were assayed for 5-HT transport activity and protein as described under Materials and Methods. Essentially identical results were obtained in three other experiments which are not shown.

column. The amount of activity which eluted with the soluble fraction, although it might seem large, represents approximately twice the background activity measured in the absence of Na^+. In other experiments the activity of pool 2 or pool 3 was variable. Some activity was always found in one of the soluble fractions but most of the transport activity always eluted with the turbid peak, pool 1. These results suggest that most, if not all, of the transporter is associated with a large aggregate when membrane vesicles are disrupted in 25 mM cholate.

Polyacrylamine gel electrophoresis in the present of sodium lauryl sulfate reveals only minor differences, if any, in protein composition between the three protein fractions from cholate–Sepharose chromatography (data not shown). The lack of enrichment of proteins in pool 1 suggests that fractionation of cholate-disrupted platelet plasma membranes on Sepharose depends primarily on the degree of protein and lipid association.

Discussion

Platelet plasma membrane vesicles are apparently completely denatured in 25 mM cholate. The 5-HT transporter, however, is not irreversibly inactivated, since transport activity is regained when cholate-disrupted membrane vesicles are reconstituted with exogenous phospholipids. Whether the transporter is reversibly denatured in cholate is unclear, however, since at present there is no method with which to measure its activity when it is not in an intact membrane.

In the experiment shown in Figure 2, most of the transport activity of native vesicles is retained in reconstituted proteoliposomes, although it is likely that reconstitution randomizes the orientation of the transporter. In all of the experiments presented here, 5-HT transport is driven by the asymmetric distribution of ions which is imposed across the membrane. Since most of the activity is recovered, it might be concluded that transporter orientation in the membrane is not crucial for function, and that the transporter is functionally symmetrical. There is no evidence that secondary active transport systems, such as the 5-HT system, function unidirectionally. In fact, evidence to the contrary exists for both bacterial and mammalian systems (Lancaster & Hinkle, 1977; Crane, 1964; Hajjar et al., 1970). An alternative explanation is that the transporter only moves 5-HT in one direction, and that it is

distributed in a random orientation in native plasma membrane vesicles, due to inversion during lysis. This is less likely since transport of 5-HT is readily reversible by dissipating the Na^+ and K^+ gradients with gramicidin (Rudnick, 1977), suggesting that the transporter functions in both directions.

The ability of soybean phospholipids to reconstitute 5-HT transport activity might suggest that transport is insensitive to the phospholipid composition of the membrane. This is not necessarily the case, however, since essential lipids could remain associated with the transporter in cholate or, alternatively, soybean phospholipids might fortuitously satisfy the lipid requirement. The association of 5-HT transport activity with the high molecular weight peak on Sepharose 6B suggests that lipids remain associated with the transporter in cholate. Moreover, the density of this particulate material is low, since it does not sediment when centrifuged for 1 h at 100 000*g* (data not shown). The question of a specific lipid requirement for transport, therefore, remains unanswered.

It is unfortunate that cholate does not completely solubilize the 5-HT transporter. Preliminary attempts at reconstitution using other detergents which may solubilize the transporter were unsuccessful. It was initially hoped that reconstitution of transport from cholate solubilized membrane vesicles could be used as an assay during transporter purification, as Kasahara & Hinkle (1977) have used reconstitution to assay Triton X-100 solubilized erythrocyte glucose transporter. The association of 5-HT transport activity with the particulate fraction of cholate-solubilized platelet plasma membrane indicates, however, that cholate is not a suitable detergent for this procedure. The small but reproducible amount of Na^+-dependent transport activity which is apparently soluble during cholate gel filtration on Sepharose 6B could be, in fact, due to particulate transporter which is retained longer by the column due to nonspecific interactions. Alternatively, it could represent a small fraction of truly solubilized transporter released from the particulate complex. In any case, the ability of the 5-HT transporter to withstand detergent concentrations high enough to completely destroy the membrane structure suggests it is sufficiently conformationally stable that, in the future, conditions might be found to completely solubilize the transporter without irreversibly destroying its activity.

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