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Reconstitution of N^1-Methylnicotinamide and p-Aminohippuric Acid Transport in Phospholipid Vesicles with a Protein Fraction Isolated from Dog Kidney Membranes

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SUMMARY

HOLOHAN, PETER D., NESS I. PESSAH, ISAAC N. PESSAH, AND CHARLES R. ROSS: The reconstitution of N^1-methylnicotinamide and p-aminohippuric acid transport in phospholipid vesicles with a protein fraction isolated from dog kidney membranes. *Mol. Pharmacol.* **16**, 343–356 (1979).

N^1-methylnicotinamide (NMN) and p-aminohippurate (PAH) transport can be reconstituted in phospholipid vesicles by incorporating the solubilized membrane proteins into artificial phospholipid membranes. After solubilization with the non-ionic detergent, Lubrol WX, the protein solution was added to a phospholipid solution (prepared by dissolving the phospholipids in the detergents) and the phospholipid:protein vesicles were allowed to form during dialysis. Transport in these vesicles was studied with radioactively labeled NMN or PAH; the transport for NMN was 173 to 394 pmoles/mg of protein/5 min and for PAH was 263 to 505 pmoles/mg of protein/5 min. Transport into control vesicles (without protein) was less than 10% of these values. The transport of NMN and PAH displayed properties of facilitated diffusion: 1) equilibrium was reached within 5 min; 2) pH dependence (optimum for NMN was 7.4, for PAH, 7.0); 3) protein and lipid concentration dependence; 4) saturability (K_m estimated from ½ saturation was approximately 1 mM for both NMN and PAH); and 5) specificity, in that known competitors of these transport systems inhibited uptake. In addition, it appears as if specific phospholipids are required in that only sphingomyelin and phosphatidylcholine functioned in reconstituting transport. Using reconstitution as the mode of assaying the proteins through an isolation procedure after solubilization, we achieved a 45 fold purification of the NMN and PAH transport proteins. Interestingly, although our data clearly show that the two transport systems are distinct entities, the two activities copurify. Based upon these results, we propose that we have accomplished a partial purification of the "carrier" proteins involved in the renal secretion of organic compounds.

INTRODUCTION

The research effort of our laboratory has been directed toward isolating the transport proteins involved in the renal secretion of organic ions (1–4). Using an "affinity" probe and a "protection" assay we were able to partially purify an organic cation-specific binding protein from dog kidney membranes (4). This experimental approach is inherently difficult, and we decided to try to reconstitute transport in

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phospholipid liposomes as the means of quantifying the transport proteins after their release from the membrane.

Recently, reconstitution of organic solute transport in liposomes has been achieved by many different investigators (5–11). Earlier studies have had reconstituted inorganic cation transport in liposomes by incorporating the various ATPases in phospholipid vesicles (12–20). Therefore, the feasibility of reconstituting organic ion transport appeared to be excellent.

Using NMN[1] as the prototype for organic cations, and PAH for organic anions, we have successfully accomplished the reconstitution of organic ion transport in liposomes. By employing reconstitution as the means of quantifying the transport proteins, we also have achieved a partial purification of the NMN and PAH transport proteins. Interestingly, the two activities copurify, in spite of the fact that our data clearly show that the two transport systems are distinct entities.

EXPERIMENTAL PROTOCOL

Membrane fractions were obtained from the kidneys of mongrel dogs as previously described (3).

Protein fractionation. We have reported on the purification of an organic cation-specific binding protein from dog kidney (4). In those studies we employed a protection or indirect binding assay to quantitate the protein after its release from the membrane. Since one of our primary interests was to re-examine the purification with respect to which protein fraction reconstituted transport, we repeated the purification steps as described (4). Therefore, complete details will not be given here, but a synopsis is as follows: After solubilization, the proteins were fractionated by DEAE-Sephadex chromatography. Five major peaks were recovered, and the one that displayed NMN binding (Peak I) eluted with the column void volume. Peak I was further fractionated by preparative-disc electrophoresis, yielding three peaks with the NMN binding was found in peak B. This latter fraction was subjected to gel filtration on Agarose A 1.5 m, giving two protein peaks. The peak displaying NMN binding was retained by the gel matrix. The homogeneity of the protein samples was examined by analytical polyacrylamide gel electrophoresis (21) and the gels were stained for protein with Coomassie blue (22), carbohydrate (23), and for lipid with oil red O (24). The samples were also examined by SDS gel electrophoresis by the method of Weber and Osborn (25).

Lipid fractionation. The polar lipids were extracted from the kidney membranes by the method described by Fleischer and Fleischer (26), and were stored in chloroform:methanol (2:1) under N_2 until used. The lipids were fractionated on hydroxylapatite by the method of Slomiany and Horowitz (27), were concentrated and were stored under N_2. We routinely recovered from the hydroxylapatite column 85% of the total phosphorous, measured by the method of Chen *et al.* (28). The identity of the lipid present in each fraction was determined by TLC by comparison with standards. Thin layer chromatography was carried out on silica gel sheets (heat activated at 120° for 30 min) with a solvent system of chloroform:methanol:H_2O (65:25:4). After development of the chromatogram, phospholipids were detected with a phosphate spray, amino groups with ninhydrin reagents, and neutral lipids with sulfuric acid-dichromate (the spray reagents were obtained from Supelco, Inc., Bellefonte, P.A.).

Preparation of phospholipid vesicles. The procedure basically consisted of solubilizing the polar lipids in a detergent solution, and then allowing phospholipid vesicles to reform, in the presence or absence of added protein, during a dialysis step. The details are as follows: An aliquot of a lipid solution was dried under N_2, redissolved in a minimal volume of diethyl ether and redried (to remove residual chloroform). The lipid was then solubilized in 25 mM sodium phosphate buffer, pH 7.0, containing 1.2% Lubrol to give a final concentration of 30 mg phospholipid/ml. The phospholipid sample was then diluted 1:1 with either a

[1] The abbreviations used are: NMN, N'-methylnicotinamide; PAH, p-aminohippurate; TLC, thin layer chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid;

protein solution (1 mg/ml) or with buffer, and was dialyzed for 48 hours at 3° against 25 mM sodium phosphate buffer, pH 7.0, 0.1 mM dithiothreitol.

The polar lipids were not completely soluble in the detergent solution; therefore, the insoluble material was removed, either before or after the dialysis, by centrifugation at 40,000 × *g* for 30 min. The supernatant fractions after the centrifugation were used in the assays.

Assay. The method is a modification of the one reported by Lefkowitz *et al.* (29). The complete details of the modifications have been previously reported (3), but for the readers convenience a brief description will be given here. The underlying principle is: The radioactive material that is transported into the liposomal vesicles can be separated from that remaining outside by centrifugation through a gel matrix.

Unless otherwise stated, the assay consisted of the following: the reaction was initiated by adding 50 μl of a liposomal suspension to a 300 μl solution containing radioactively labeled NMN or PAH in a 25 mM sodium phosphate buffer, pH 7.0. All the reactants were temperature equilibrated at 37° before initiating the reaction. After a 5 min incubation period, triplicate 100 μl samples were removed and applied to top of a 3 ml Sephadex G-25 gel column and were "washed in" with two successive applications of 100 μl of 25 mM sodium phosphate buffer, pH 7.0. After the sample application, the columns were centrifuged at 10,000 × *g* for 7 min. The entire column eluate was collected in a scintillation vial, 10 ml of Instabray added, and the amount of radioactivity taken up by the vesicles was determined by standard liquid spectrophotometric techniques. Controls (minus the protein) were always run simultaneously in order to check the retention capacity of the columns and as correction for background. The method of preparing the columns, and the means of positioning them over the scintillation vials was described previously (3).

Centrifugation through the gel matrix has proven to be a very effective means of separating the vesicles that appear with the column void volume from the free radioactivity that is trapped within the gel (3). More than 85% of the protein applied to the column can be recovered while as much as 1 μCi can be retained by the gel matrix (3).

Chemicals. The routine chemical reagents used in these experiments were of the highest purity commercially available and were not further purified before use. [^{3}H]NMN, specific activity of 1.11 Ci/mmol, was custom synthesized by International Chemical and Nuclear Corporation (Irvine, Calif.); purity was ascertained by TLC before use. [^{3}H]PAH (247 mCi/mmole) was obtained from Amersham/Searle (Arlington Heights, Illinois); and [^{14}C]PAH (28.9 mCi/mmole) from New England Nuclear (Boston, Mass.).

The Sephadex G-25 was obtained from Pharmacia Fine Chemical Inc. (Piscataway, N.J.). The liquid scintillant was Instabray, obtained from Yorktown Research (New Hyde Park, N.Y.). Phospholipid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Reconstitution of transport. The supernatant fraction obtained by solubilizing dog kidney membrane (see EXPERIMENTAL PROTOCOL) was added to a polar lipid solution and was subjected to a dialysis procedure as described (see EXPERIMENTAL PROTOCOL). After dialysis, the contents of the dialysis sack were assayed for organic ion uptake as described above. The results of a typical experiment are listed in Table 1. Routinely, the amount of radioactivity found in these samples to which the lipid was added was 10 to 12 fold higher than the controls which consisted of solubilized membranes diluted 1:1 with 25 mM sodium phosphate buffer, pH 7.0 containing 1.2% Lubrol and dialyzed as above.

Our interpretation of these data was that the carrier proteins (released by the solubilization procedure) were reconstituted into liposomes, and therefore, the results reflect NMN and PAH transport into these vesicles. The uptake obtained for those samples where lipid was not added back (controls) probably represents transport also, since a few small vesicles were still

TABLE 1

Reconstitution of NMN and PAH transport

Solubilized dog kidney membranes were dialyzed in the presence or absence of polar lipids and assayed for transport (see EXPERIMENTAL PROTOCOL for details). The final concentration of either [^{3}H]NMN (275 mCi/mmole) or [^{3}H]PAH (210 mCi/mmole) was 50 μm. The results are expressed as the amount of radioactivity appearing in the column void volume (see EXPERIMENTAL PROTOCOL) per mg of protein present in the reaction solution. The data are the means of triplicate determinations.

	Uptake	
	NMN	PAH
	(dpm/mg protein/5 min × 10^3)	
Solubilized membranes		
Without lipid	12.9	8.6
With membrane lipid[a]	138.4	106.2
Cytosol[b]		
Without lipid	1.6	1.3
With membrane lipid[a]	1.5	1.0

[a] Lipid refers to polar lipids extracted by the method of Fleischer & Fleischer (26) from dog kidney membranes isolated as previously described (3).

[b] Cytosol refers to cytoplasmic proteins not pelleted at 40,000 × g for 60 minutes and which were diluted with buffer containing detergent before dialysis (see Results for details).

present in supernatant fraction after membrane solubilization (see explanation below).

As an additional control, the cytosol (that portion of the homogenate not pelleted at 40,000 × *g* for 60 min) was diluted with buffer containing 1.2% Lubrol to the same protein concentration as the solubilized membranes, and was treated as above. As the results in Table 1 show, cytosol proteins did not reconstitute transport. The radioactivity found with this fraction may reflect the specific binding of PAH to ligandin (30) and NMN to an as yet unidentified protein(s).

While reconstitution experiments as described above were successful on each attempt, there was considerable quantitative variation. For example, with nine different membrane preparations the reconstituted NMN transport varied from a low value of 173 pmoles/mg of protein/5 min to a high of 394 pmoles/mg of protein/5 min while the reconstituted PAH transport varied from 263 to 505 pmoles/mg of protein/5 min (using a final concentration of either NMN or PAH of 50 μM). The values given are the mean of triplicate determinations. Although the triplicates varied by less than 10% from the mean, the results show the large variation in the extent of reconstitution obtained in different experiments, illustrating one of the inherent problems associated with the methodology. Two possible explanations for this variation are (1) different amounts of the transport proteins were reincorporated into liposomes or (2) the actual number of liposomes formed during the dialysis procedure varied. In order to minimize the latter possibility, the ratio of unfractionated phospholipid to protein at the start of the dialysis was always 30:1 (mg/mg), a ratio that was found to produce the maximal effect (data not shown). Therefore, we believe a major reason for the variation was due to differing amounts of protein in the liposomes. In support of this interpretation was the finding that the reconstituted NMN and PAH transport correlated with one another, that is, when NMN transport was low, so was PAH.

Criterion for vesicle formation. Transport implies a specific binding of the substrate to the transport protein followed by translocation across the membrane into an intravesicular space. The following functions were selected to assist in evaluating whether or not vesicles were present.

a. Electron microscopy. Experimental (phospholipid plus solubilized protein) and control (phospholipid alone) solutions were subjected to a dialysis procedure as described (see EXPERIMENTAL PROTOCOL). The contents of the dialysis bags were centrifuged at 250,000 × *g* for 1 hour, yielding pellet fractions that were resuspended in 25 mM Tris-HEPES buffer, pH 7, and examined by electron microscopy after negative staining with phosphotungstic acid. The results show that vesicles were present in both the control (Fig. 1B) and experimental, i.e., reconstituted (Fig. 1C), samples, although there was a wide distribution in vesicle size, especially in the reconstituted system. Perhaps this observation explains in part the variation in the amount of organic ions transported as described above.

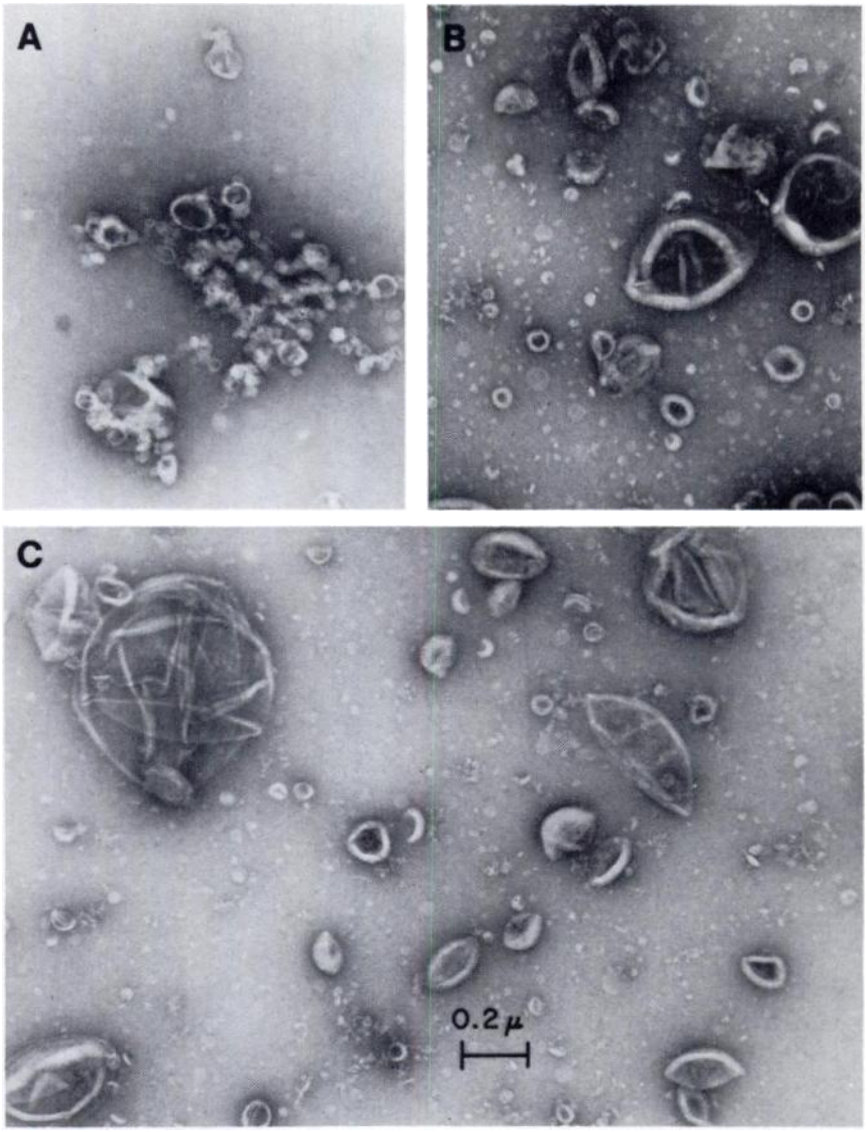

FIG. 1. *Electron micrographs of solubilized kidney membranes (A); control phospholipid vesicles (B); and reconstituted liposomes (C)*

The samples were prepared as described in EXPERIMENTAL PROTOCOL and were viewed by a negative staining technique.

The solubilized protein was diluted with the Tris-HEPES buffer to the same protein concentration (1 mg/ml) that was present in the resuspended pellet fraction from the experimental sample and was examined by electron microscopy. The results (Fig. 1A) reveal the presence of small vesicles within the sample. Their presence probably explains the uptake observed with this material (Table 1, solubilized protein without added lipid). These results also provided a criterion for accepting "reconstitution of transport": the transport obtained for samples to which lipid had been added prior to dialysis must be significantly greater than those without lipid.

These experiments provided the opportunity for an additional test for reconstitution of transport, that is, the supernatant and the resuspended pellet from the exper-

imental sample (soluble protein plus phospholipid) were assayed, and the transport capacity was quantitatively recovered in the pellet fraction, indicating that the protein was no longer in a "soluble" state but rather was associated with phospholipid (data not shown). A similar technique has been reported by others (31) for the isolation of liposomes.

b. Effect of osmotic pressure. Under the experimental conditions used, any maneuver affecting the intravesicular volume should have an effect on transport. Therefore, various amounts of mannitol were added to the assay solution and its effects noted. As the results show (Table 2) increasing concentrations of mannitol decreased the uptake of both NMN and PAH. If the data reflected binding rather than transport, one would not expect to observe such dramatic changes in uptake as the intravesicular volume decreased. Another test for the presence of vesicles was the following: the experimental and control samples were dialyzed against distilled water (pH 7) overnight and then assayed for transport. This treatment destroyed all the transport capacity.

Properties of Transport. If specific transport protein were inserted in the phospholipid liposomes during the dialysis procedure, then the observed transport should display the properties of facilitated diffusion. Therefore, the following battery of experiments was carried out.

a. Time. Equilibrium was attained within 5 min for both NMN and PAH when used at a 50 μM concentration. At a higher concentration (14 mM) equilibrium was reached more quickly (approximately 2 min). For the control phospholipid vesicles, the time required to come to equilibrium was greater than 30 min.

b. pH. Apparent pH optima were observed for NMN and PAH transport (Fig. 2B); 7.0 for PAH and 7.4 for NMN. If the data were due to leak, then the uptake of PAH should increase as the pH was lowered (pK_a for PAH is 3.8), while the NMN (a quarternary nitrogen compound) leak should be independent of pH. These were the findings obtained with control vesicles (data not shown). However, the data (Fig. 2B) showing the dramatic effect of pH had on the uptake were what one would have expected if transport proteins had been incorporated in the liposomes.

c. Protein concentration. Transport was dependent on the concentration of vesicles (Fig. 2C), thus a function of the protein concentration. Fig. 2C illustrates that the rate of transport increased in proportion to the concentration of reconstituted vesicles added to the reaction solution. The observed increase was significantly greater than that recorded for the control phospholipid vesicles.

d. Saturation. If the results reflect facilitated diffusion, then the system should be saturable. As the results show (Fig. 2D), saturation was approached, but not attained. Our explanation for this observation is that the organic ions accumulated within the vesicle came via two routes: translocation by the transporter and by leak. The transporter becomes saturated as the concentration of substrate (NMN or PAH) is increased, but leak is a nonsaturable process, and therefore, when both processes are present, saturation is only approached. Assuming the concentration for half-saturation measures K_m, then the K_m for both

TABLE 2

Effect of osmotic pressure on NMN and PAH transport

Reconstituted liposomes were prepared (see EXPERIMENTAL PROTOCOL for details) and were added to various concentrations of Mannitol (the values listed refer to final concentrations). After allowing the solutions to incubate for 10 min, the transport assay was initiated by adding either [^{3}H]NMN (275 mCi/mmole) or [^{3}H]PAH (210 mCi/mmole) to the reaction solution. The effect of increasing osmotic pressure was compared to reconstituted vesicles that were incubated in 25 mM sodium phosphate buffer. The results are expressed as percent of control and are the means of triplicate determinations.

Additions of mannitol	Transport	
	NMN	PAH
	(% control)	
None	100	100
125 mM	82	76
166 mM	63	59
250 mM	44	43
500 mM	26	27

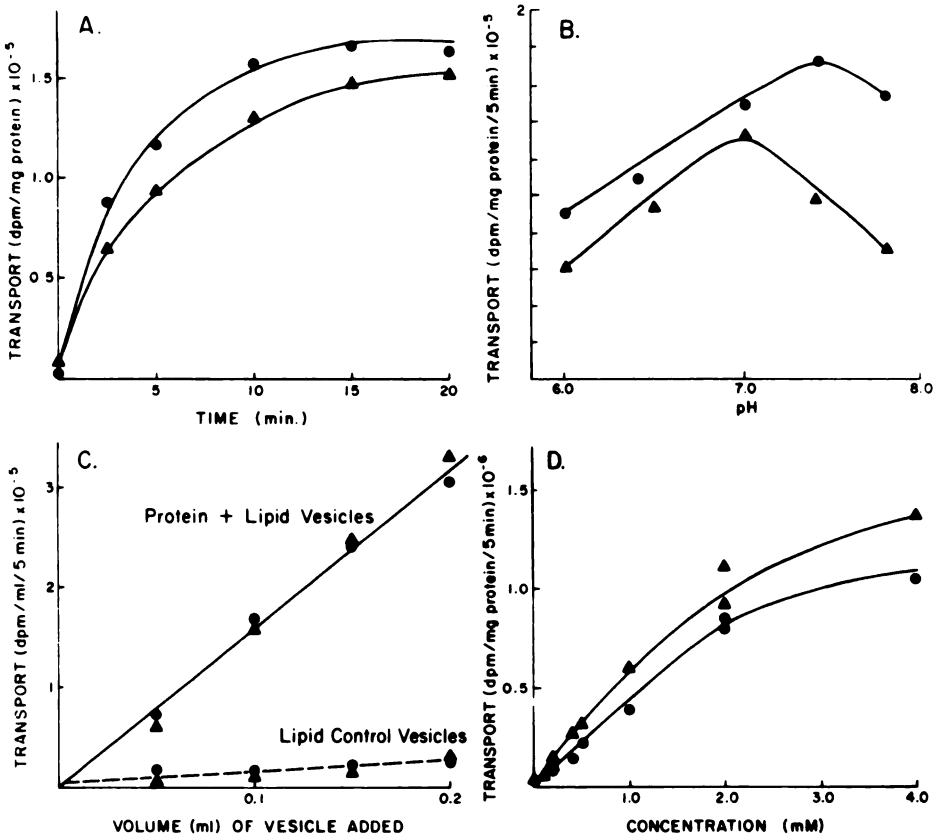

FIG. 2. *The effect of time (A); pH (B); protein concentration, expressed as volume of liposomes added (C); and external organic ion concentration (D) on the transport of NMN and PAH*

The reconstituted vesicles were prepared and used to follow transport as described in EXPERIMENTAL PROTOCOL. For these reconstitution experiments the membrane polar lipid fraction was employed. (With the exception of the data given in C,) all transport values were corrected for the simple diffusion of NMN (●) and PAH (▲) into control phospholipid vesicles which were put through the identical experimental manipulations.

NMN and PAH is approximately 1 mM, a value somewhat higher than the literature values for transport of these ions in tissue slices (K_m for PAH, 0.3 mM (32), and NMN, 0.5 mM (33).

e. Specificity. The specificity of transport was tested and the results are listed in Table 3. NMN and PAH do not mutually interfere with one another at low concentrations (50 μM), likewise NaCl has no effect on either transport systems at 5 mM. The addition of unlabeled NMN decreased the transport of [^{3}H]NMN, but also had some effect on PAH transport. Darstine (an inhibitor of organic cation transport) characteristically lowered the transport of NMN, but also affected PAH. A similar situation was observed for the organic anion transport. These data were obtained at an equilibrium state, and since electrical neutrality must be maintained, the inorganic counter ion that presumably moves with one organic ion could inhibit the transport of the other organic ion.

Previously, we had shown that dibenamine was a specific inhibitor of organic cation transport, but not for organic anions (1–4). As shown in Table 4, this pattern for dibenamine inhibition holds with the reconstituted system, and was observed over a concentration series of dibenamine. While the maximum inhibition of NMN transport was not as great as that found with tissue slices (1, 2), it was of the same order of magnitude as that found with isolated membranes (3). Our suspicion is that the

TABLE 3

Specificity of NMN and PAH transport

Transport of [^{3}H]NMN (79 mCi/mmole) and [^{14}C]PAH (39 mCi/mmole) was measured as described in EXPERIMENTAL PROTOCOL. For these experiments the final concentration of NMN and/or PAH was 100 μm. The reaction was initiated by adding the reconstituted liposomes to a solution containing the radioactivity labeled substrate in the presence or absence of competitors with a final concentration was 5 mM.

Organic ion	Competitor	Transport	
		NMN	PAH
		(% control)	
[^{3}H]NMN		100	—
[^{14}C]PAH		—	100
[^{14}C]PAH + [^{3}H]NMN		98	101
[^{3}H]NMN + [^{14}C]PAH	5 mM NMN	77	88
[^{3}H]NMN + [^{14}C]PAH	5 mM Darstine	36	62
[^{3}H]NMN + [^{14}C]PAH	5 mM PAH	91	68
[^{3}H]NMN + [^{14}C]PAH	5 mM Probenecid	86	28
[^{3}H]NMN + [^{14}C]PAH	5 mM NaCL	99	101

noninhibitable NMN uptake (Table 4) reflects leak.

Isolation. We were primarily interested in verifying our previous isolation scheme where an indirect binding assay was used to quantify the NMN binding protein (4), and therefore the complete details of the chromatographic steps will not be repeated here (a synopsis was given in the experimental section).

The starting material was 225.6 mg of solubilized membrane protein (approximately 50% of the total membrane protein), and it displayed the capacity to reconstitute NMN and PAH transport (Table 5). This material was subjected to chromatographic analysis and the resultant protein fractions were tested for their capacity to reconstitute transport. As the results show (Table 5) only Peak I gave activity. Peak I was chromatographed further and all fractions were tested for reconstitution of transport. Throughout the chromatographic steps only one faction produced reconstitution (Table 5) and the chromatography pattern showing reconstitution of NMN transport was identical to that one for the isolation of a NMN-specific binding protein (4). However, a surprising complication was that the PAH transport protein copurified with the NMN transport protein (Table 5).

By defining a unit of transport as: 1 nmole of organic ion transported/mg of protein/5 min, it was possible to determine

TABLE 4

Effect of dibenamine on NMN and PAH transport

Reconstituted phospholipid vesicles were prepared as described (EXPERIMENTAL PROTOCOL) and aliquots were incubated in the presence of various concentrations of dibenamine for 30 minutes at 37°. After the incubation period the vesicles were assayed for [^{3}H]-NMN (79 mCi/mmole) and [^{14}C]PAH (39 mCi/mmole) transport as described (EXPERIMENTAL PROTOCOL). The final concentration of NMN and PAH was 100 μM. The results are expressed as percent of controls, that is, the transport measured in reconstituted vesicles that were exposed to the identical concentrations of ethyl alcohol (the vehicle for dibenamine) as were the experimental.

Additions	Concentration	Transport	
		NMN	PAH
		(% of Control)	
None		100	101
Dibenamine	0.1 μM	89	98
"	0.5 μM	84	100
"	1.0 μM	78	98
"	50 μM	40	96
"	84 μM	35	101
"	100 μM	32	102

the extent of purification. The NMN transport protein was purified 44 fold while the purification of the PAH transporter was 47 fold (Table 5). Likewise, the yield of the final product (Peak 2 from the gel filtration) was 5% (11 mg) of the starting material. The values listed in Table 5 are means of triplicate determination. In order to test

TABLE 5

Purification of the NMN and PAH transport proteins

Dog kidney membranes were solubilized with the non-ionic detergent Lubrol WX as described (4). Solubilization released 49.5% of the membrane protein into the supernatant fraction which was used as the starting material for the purification procedure. The details of the isolation steps were published previously (4), and were briefly outlined in EXPERIMENTAL PROTOCOL. Reconstitution was performed as described (EXPERIMENTAL PROTOCOL) in which the unfractionated polar lipids were used at a lipid/protein ratio of 30:1 (mg/mg). Transport was measured with either [^{3}H]NMN (275 mCi/mmole) or [^{3}H]PAH (210 mCi/mmole) at a final concentration of 50 μM. The data were corrected for any leak of either NMN or PAH by subtracting the uptake found with control liposomes (prepared as described EXPERIMENTAL PROTOCOL). The results are expressed as nmoles/mg of protein present in the reaction solution and are the means of triplicate determinations.

Fraction	Vol	Protein	Transport NMN	PAH
	(ml)	*(mg/ml)*	*(nmoles/mg protein/5 min)*	
Solubilized membranes	48	4.7	0.26	0.21
DEAE Sephadex				
Peak I	12	3.0	3.02	2.56
Peak II	5	0.6	0.08	0.06
Peak III	20	4.0	0.01	None
Peak IV	5	1.6	None	0.01
Peak V	15	3.5	<0.01	None
Prep-Disc				
Peak A	5	0.2	None	None
Peak B	18	0.9	7.63	8.97
Peak C	12	0.8	0.04	<0.01
GEL-Filtration				
Peak 1	5	0.2	None	None
Peak 2	14	0.8	11.44	9.83

the reproducibility of the transport capacity, three different reconstituted liposomal preparations were examined and the transport was found to be 11.3 ± 1.4 nmoles of NMN/mg of protein/5 min, and 10.5 ± 2.2 nmoles/mg of protein/5 min for PAH.

As additional support for the validity of the isolation procedure, five purifications have been carried to completion and the transport capacity of the final product was 11.5 ± 1.9 nmoles NMN/mg of protein/5 min (S.D., $n = 5$), and 10.1 ± 1.7 nmoles PAH/mg of protein/5 min (S.D., $n = 5$).

Again in each purification, the NMN and PAH proteins copurified.

Purity of transport proteins. Previously, we had shown that the protein fraction obtained after the gel filtration step gave a single, although diffuse, band on polyacrylamide gels, and that it gave a positive stain for protein, carbohydrate, and lipid (4). We have repeatedly observed this phenomenon, and in addition, have found that electrophoresis at pH 3.6 gave a single, slowly migrating, diffuse band. Since a lipid staining material was still present, we assumed that we had isolated a lipid:protein:detergent complex, and attempted to disrupt the complex with sodium dodecylsulfate (SDS). Analysis of the material after SDS gel electrophoresis showed a complex pattern (4), and that lipid was still present (4). Therefore, SDS gel electrophoresis was performed after the lipid was extracted as follows: the protein (gel filtration, Peak 2: Table 5) was dialyzed against H_2O for 24 hours, freeze-dried, and extracted three times with either chloroform:methanol (2:1) or methanol:acetone (1:1). The resultant insoluble material (collected by centrifugation) was dried under N_2 and was dissolved in SDS buffer, and was analyzed by SDS gel electrophoresis. As shown (Fig. 3) there were at least nine polypeptides present (gel 2), with two carbohydrate positive zones (gel 1): a large molecular weight glycoprotein, and the other an unidentified component (which migrated faster than the tracking dye). Thin-layer chromatographic analysis of the organic solvent extracted mate-

FIG. 3. *SDS gel electrophoresis of partially purified transport proteins (Gel-Filtration, Peak 2)*

The electrophoresis was carried as described (4) on that material which was found to be organic solvent insoluble (see RESULTS). After electrophoresis the gels were stained for protein (Gel 2) and carbohydrate (Gel 1) as described previously (4).

rial showed the presence of only phosphatidylcholine and sphingomyelin, with no ninhydrin positive components. Transport could not be reconstituted after the organic solvent, SDS treatment.

Specificity of phospholipids. The membrane lipids were separated on hydroxylapatite (see EXPERIMENTAL PROTOCOL) and the resultant fractions were tested for their capacity to reconstitute transport (Table 6). Only Fractions III and IV functioned and their contents were identified by thin-layer chromatography as phosphatidylcholine (Fraction III), and phosphatidylcholine and sphingomyelin (Fraction IV). The lipid/protein ratio listed in Table 6 represents that which was present at the start of the reconstitution procedure and not the actual amount reconstituted into liposomes. Reconstitution was based upon whether the data obtained with the lipid fractions were greater than those found with the solubilized membranes without lipid (Table 1).

To examine further the specificity of the lipid moiety, a series of commercially prepared phospholipids were examined (Table 7). In the L-α-phosphatidylcholine series, dioleoyl and soy bean were about as equally effective as Fraction III and egg yolk was approximately one-third as effective, whereas those containing only saturated fatty acid side chains were totally ineffective in reconstituting transport. (It is possible that the effects seen merely reflect a difference in the phospholipid solubility.) Neither L-α-lysophatidylcholine (soy bean), nor phosphatidic acids reconstituted transport, a finding consistent with their known water solubilities. The L-α-phosphatidylethanolamine series was ineffective in reconstituting transport. Again those data which were not greater than the solubilized membranes without lipid (Table 1) were taken as nonfunctional for reconstitution.

Sphingomyelin from bovine brain was only 20% as effective as was Fraction III; however, the sphingomyelin from dog kidney microsomes (separation of Fraction IV by preparative TLC) appears to be as equally effective as was phosphatidylcholine (data not shown).

It is possible that at high enough concentrations those phospholipids that did not reconstitute transport would do so. However, we have been unable to observe this. In fact, a concentration series of the various phosphatidylcholines was examined, and the order of effectiveness listed above still held with the maximal effect of each being at a ratio of lipid to protein of 2.5 to 1. One difficulty with these experiments is that the liposomes were formed at 3°. It is possible that at higher temperatures the saturated phospholipids would function. This possibility remains to be examined.

TABLE 6

Capacity of lipid fractions resolved by hydroxylapatite chromatography to reconstitute NMN and PAH transport

The polar lipids extracted from dog kidney membranes by the method of Fleischer and Fleischer (26) were fractionated on hydroxylapatite by the method of Slomiary and Horowitz (27). The resultant fractions were compared to the unfractionated material for their capacity to reconstitute transport. The reconstituted phospholipid vesicles were prepared as described and were used to measure either [^{3}H]NMN (275 mCi/mmole) or [^{3}H]PAH (210 mCi/mmole) transport (see EXPERIMENTAL PROTOCOL for details). The lipid/protein ratio refers to that present at the start of the reconstitution experiments and not the actual amounts reconstituted into vesicles. The amount of neutral lipids was determined by dry weight while phospholipids were calculated by phosphate analysis (28). The values listed were the mean of triplicate determinations and were not corrected for leak.

Additions	Transport		Lipid/protein	Content
	NMN	PAH		
	(dpm/mg protein/5 min) × 10^3		*(mg/mg)*	
Unfractionated lipid	139.9	113.4	30.0	—
Fraction I	6.7	2.3	12.0	Neutral lipids
Fraction II	6.6	2.0	12.4	Neutral lipids
Fraction III	133.1	99.1	9.5	Phosphatidylcholine (PC)
Fraction IV	92.5	96.8	9.4	PC; sphingomyelin
Fraction V	4.2	2.4	9.8	Phosphatidyl/ethanolamine (PE)
Fraction VI	3.1	2.0	7.6	PE
Fraction VII	3.0	1.4	32.8	Phosphatidyl serine, lyPC plus undetermined

DISCUSSION

The proximal tubule cells of the mammalian kidney actively secrete organic cation and organic anions by separate and distinct systems (for reviews see 34–39). Since these are active transport processes, it is assumed that the translocation of the organic ions across the membrane is catalyzed by specific transport proteins (transporters). In previous reports from our laboratory we have demonstrated their presence (1–3), and were able to effect a partial purification of an organic cation-specific binding protein from dog kidney membranes (4).

As the results in this report show we confirmed and extended our previous results by successfully reconstituting organic anion and cation transport in liposomes. The data are more consistent with transport into the osmotically active space of the liposomes than binding to the phospholipid matrix (Fig. 1 and Table 2). The fact that the observed uptake displayed properties of facilitated diffusion (saturable, Fig. 2D; and blocked by specific inhibitors, Tables 3 and 4) was taken as evidence that specific transport proteins were incorporated in the liposomes.

While the results confirmed our previous isolation of an NMN-binding protein (4), they uncovered another complication. The NMN and PAH transport proteins copurified (Table 5). Although both transporters were within the same protein fraction, they were independent of one another (Tables 3 and 4). We suspect that we have isolated a protein:lipid:detergent complex and therefore, the association of these proteins might be an artifact of the solubilization and isolation procedure. However, two arguments against this are: first, in every isolation experiment performed the two transport systems always appeared together (whether or not detergent was kept in the buffer system), and second, both copurified after solubilization with deoxycholate (unpublished results). Therefore, it is possible that these transport systems are topographically associated but physiologically independent (Tables 3 and 4). We anticipate that some additional step will disrupt the complex (without denaturating the proteins) and

TABLE 7

Effect of various phospholipids on NMN and PAH transport

A series of phospholipids were tested for their capacity to reconstitute either NMN or PAH transport using solubilized unfractionated kidney membranes as the source of the transport proteins. The reconstituted vesicles were prepared and used for the transport assay as described. The final concentration of either NMN or PAH was 50 μM. The lipid/protein ratio (mg/mg) refers to that present at the start of the dialysis procedure.

Lipid added	Transport		Lipid/Protein
	NMN	PAH	
	(dpm/mg protein/5 min) × 10^3		*(mg/mg)*
Fraction III	118	100	4.3
1-α-phosphatidyl cholines:			
Dilauroyl (12:0)	4	3	3.4
Dimyristoyl (14:0)	5	4	3.4
Dipalmitoyl (16:0)	5	3	3.4
Distearoyl (18:0)	5	2	3.4
Dioleoyl (18:1)	114	104	3.4
Egg yolk	42	29	3.4
Soy bean	110	106	3.4
L-α-lysophosphatidyl choline:			
Soy bean	3	2	3.4
L-α-phosphatidyl ethanolamines:			
Dipalmitoyl (16:0)	11	4	3.4
Bovine brain	8	3	3.4
E. coli	10	4	3.4
Sphingomyelin			
(Bovine brain)	21	17	3.4
Phosphatidic acid			
Dipalmitoyl (16:0)	3	3	3.4
Soy bean	3	2	3.4

will allow for the separation of the NMN and PAH transport proteins, although in preliminary experiments neither chaotropic reagents nor urea have proven successful.

Current models for transport argue against the concept of mobile carriers, and against transport proteins rotating within the membrane, especially if they are glycoproteins (40). Only one glycoprotein staining zone was found after SDS-gel electrophoresis (Fig. 3). The data, however, are not sufficient to establish whether or not it represents a single polypeptide. Possibly several glycopeptides of the same molecular size are present. If this were the case, then the transport system might consist of a glycoprotein core that could act as a channel with the smaller polypeptide some how functioning in recognition of the permeat ion. Since these transport systems are pleiotropic in nature (34–39), it is possible also that a family of glycoproteins exist which are the channels through which the organic ions move.

Establishing the chemical properties of transport proteins will aid immeasureably in understanding the molecular events underlying transport processes. Reconstitution of transport provides the means by which these proteins can be identified throughout an isolation scheme, thereby, making their isolation possible. It is a new technique and information from many different laboratories will have to be compiled before standardized methods are prescribed. With our method, reconstitution of NMN and PAH transport was successfull in every attempt. However, as noted, there was a significant variation among the preparations. Probably we can lessen this variation somewhat by collecting the liposomes by centrifugation before assaying them. While we carefully controlled the ratio of phospholipid to protein for the formation of the liposomes, we were unable to determine this ratio accurately in the reconstituted vesicles because the experiments were performed in a phosphate buffer. Perhaps by changing buffer systems, this data can be compiled and evaluated.

Since we have not established if the reconstituted liposomes are uni- or multilaminar vesicles, it seemed unwise to attempt kinetic measurements at this time. Therefore, our gel column method (3) was used to separate the "free" and "transported" material. Since several minutes are required to complete the assay, the studies were carried out by letting the system come to equilibrium; therefore, the results were presented as transport/mg of protein/5 min. The very important problem of evaluating the kinetic parameters will have to await the resolution of these problems.

Based upon some unpublished results showing that the transport of the organic ions in membrane vesicles was sensitive to

phospholipase A (or C) digestion, we anticipated that very specific lipid-protein interactions were involved in the transport processes. It was for this reason that we selected the phospholipids present in the dog kidney membranes themselves as the lipid source for the reconstitution experiments. As results show, they functioned efficiently. Fractionation of the lipids from dog kidney membranes indicated some specificity as to which ones would function (Table 6). However, Tyrell *et al.* (41) reported that not all phospholipids form liposomes, and this may explain the apparent specificity observed (Table 6). Although, when a series of phosphatidylcholines was examined, differential effects were observed (Table 7). Again it is difficult to arrive at an unequivocal interpretation of these results since we did not establish whether or not vesicles were formed in each instance. Therefore, the lipid specificity remains an open question and will have to be explored in greater depth.

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