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The Purification of an Organic Cation-Specific Binding Protein from Dog Kidney

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SUMMARY

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A carrier-mediated mechanism has been postulated for the renal transport of organic cations. An indirect assay, based upon the capacity of an organic cation, N^1-methylnicotinamide, to protect against [^{14}C]dibenamine alkylation, was used to quantitate the carrier protein during the isolation procedures. Following solubilization from kidney membranes with the nonionic detergent Lubrol WX, the carrrier protein was purified to apparent homogeneity as indicated by polyacrylamide gel electrophoresis. The protein has both lipid and carbohydrate associated with it. Sodium dodecyl sulfate (SDS)-gel electrophoresis separated the components, with the bulk of the material appearing as a carbohydrate-lipid-protein complex that migrated faster than the tracking dye. In the SDS-gel experiments [^{14}C]dibenamine was found to be covalently bound at a narrow locus, and this binding was "protected" by N^1-methylnicotinamide. The data are insufficient to prove that the isolated protein is the carrier for organic cations, but are consistent with this interpretation.

INTRODUCTION

Both exogenous organic cations (drugs and detoxified products produced by the liver) and endogenous ones (naturally occurring metabolites) are actively secreted by the proximal tubule cells of the mammalian kidney (for reviews, see refs. 1-6). A characteristic of this secretory system is that it possesses high efficiency but low specificity; that is, many cations are believed to be transported by the same carrier (7-10).

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With the aim of isolating and characterizing the cellular components involved in this process, we first envisioned a simple model for transport: (a) initial binding of the ion to the carrier protein, (b) translocation across the membrane, and (c) release of the transported cation. Utilization of an affinity reagent that specifically and irreversibly binds to the carrier should therefore permit its isolation. This general approach has been summarized by Mautner (11).

In initial experiments, Ross *et al.* (12) observed that β-haloalkylamines (dibenamine and dibenzyline), known *alpha* adrenergic blocking agents, specifically and irreversibly inhibited renal organic cation transport, but not the transport of organic anions or other cellular functions. These

results suggested that the β-haloalkylamines might form cyclic cation intermediates (13, 14), which compete with other organic cations for binding (15) and eventually inactivate the mechanism by alkylation (12). Others (16, 17) have suggested that a carbonium ion intermediate is the actual alkylating species.

These compounds provided the experimental tool for the development of an indirect binding or "protection" assay (12, 15) based on the principles enunciated by Koshland *et al.* (18). Employing this assay, Ross *et al.* (15), using dog kidney tissue, were able to localize the "protection" afforded to a protein fraction by N^1-methylnicotinamide against dibenamine inhibition.

As the results presented in this communication show, the problem has been advanced the next step. An organic cation-specific binding protein has been isolated to an apparent state of homogeneity, and, based upon its capacity to be protected, it conceivably is the carrier protein.

METHODS

Protection Assay

The percentage of protection afforded by an organic cation, NMN,[3] was calculated from the difference between the amounts of dibenamine bound in its absence and presence. Dialysis sacks were prepared from No. 8 cellulose dialysis tubing obtained from VWR Scientific (Rochester, N. Y.) after having been boiled in 1 mM EDTA and then twice again in glass-distilled H_2O. One milliliter of a protein solution (0.5–2.0 mg/ml) was placed inside the sack and dialyzed for 24 hr at 33° against a [^{14}C]dibenamine solution, 1 μM (0.3 μg/ml), containing either 10 mM NaCl, or 10 mM NMN. The [^{14}C]dibenamine solutions were prepared just before use from a stock at 50 μg/ml in 20% ethyl alcohol–0.04 N HCl.

Under these experimental conditions, dibenamine alkylation increased to a maximum (after 4 hr), with the percentage of protection remaining constant thereafter. Probably the "ethylenimmonium" intermediate was converted to an alcohol (13), thus stopping further alkylation. Additional dialysis time was required to attain equilibrium for the unbound radioactivity. After dialysis, triplicate 0.25-ml samples of both the inside and outside solutions were added to 10 ml of Instabray (Yorktown Research), and radioactivity was counted in a Nuclear-Chicago Mark I liquid scintillation counter equipped with an external standard for quenching corrections.

The radioactivity bound was expressed per milligram of protein, which was determined by the method of Lowry *et al.* (19), using BSA as standard. Absorption of dibenamine to the dialysis tubing was negligible, since the recovery of the radioactivity added was 98.05% ± 2.63% (n = 21).

Isolation Procedures

Tissues were obtained from both male and female mongrel dogs that had been maintained on a standard laboratory diet. The procedures for preparing renal cortical slices and measuring uptake have been described (8, 12, 15, 20–22).

Osmotic shock cycle. Reportedly (23) an osmotic shock cycle releases a carrier-like protein from kidney tissue slices. We modified this procedure (shown as a flow diagram in Fig. 1) as follows. Three tissue slices (approximately 100 mg each) were placed in 2 ml of a hypertonic sucrose solution (0.5 M, pH adjusted to 7.4 with 1 M Tris) for 10 min with frequent agitation, using a Vortex mixer, to yield a sucrose osmotic shock fluid. The tissue was transferred to a test tube containing 2 ml of a hypotonic $MgCl_2$ solution (0.5 mM, pH adjusted to 7.4 as above) for 30 min and again subjected to frequent agitation. The tissue residue was discarded after this step, leaving the $MgCl_2$ osmotic shock fluid.

The osmotic shock fluids were centrifuged at 40,000 × g for 30 min in a Sorval RC2B refrigerated centrifuge. The pellet (P_2) was washed by resuspension in 50 volumes of 100 mM sodium phosphate buffer, pH 7.2, using a hand-held glass homogenizer, and centrifuged as above. The final

[3] The abbreviations used are: NMN, N^1-methylnicotinamide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

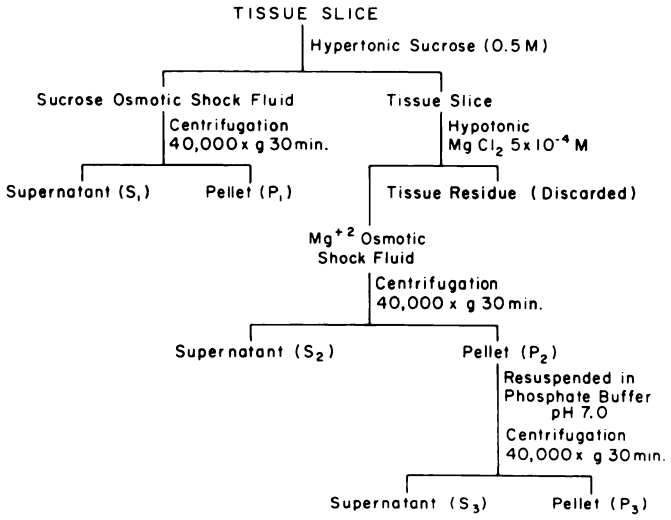

FIG. 1. *Osmotic shock cycle*

Renal cortex tissue slices were subjected to an osmotic shock cycle as described under METHODS, producing a sucrose hypertonic osmotic shock fluid and a hypotonic $MgCl_2$ osmotic shock fluid each of which in turn was separated by centrifugation into supernatant and pellet fractions.

pellets (P_1 and P_3) were resuspended in one-sixth the original volume with the above buffer.

Solubilization. The pellet (P_3) was solubilized with the nonionic detergent Lubrol WX as follows. The suspension was centrifuged at 40,000 × *g* for 30 min, and the pellet was suspended in the same volume of 100 mM sodium phosphate buffer, pH 7.4, containing 1.2% (w/v) Lubrol WX. It was transferred to a glass homogenizer and subjected to 10 strokes by hand, allowed to stand for 10 min in an ice bath, subjected to an additional 10 strokes, and then centrifuged at 105,000 × *g* for 1 hr.

DEAE-Sephadex chromatography. The ion-exchange matrix employed was DEAE-Sephadex A-50 (Pharmacia) equilibrated with 100 mM sodium phosphate buffer, pH 7.2, for at least 1 week before use. Before application to the column, the solubilized protein fraction was dialyzed against the starting buffer for 24 hr. All the isolation steps were carried out at 3–4°.

Preparative disc electrophoresis (24). The experiments were performed with a Canalco instrument, using a 5% acrylamide separating gel with an electrode buffer of 27 mM Tris-glycine, pH 8.3, and an elution buffer of 37.5 mM Tris-HCl, pH 7.4. A stacking gel was not used; instead, the sample was diluted 1:1 with a 50% acrylamide solution and then layered directly onto the separating gel. Electrophoresis was conducted at 400 V, and the instrument was cooled to 4° throughout the course of the experiment.

Gel filtration. These experiments were carried out using Agarose A-1.5 m (Bio-Rad) equilibrated with 100 mM sodium phosphate buffer, pH 7.2.

Analytical Polyacrylamide Gel Electrophoresis

The method was that of Gabriel (25), which was a slight modification of the method described by Davis (26). The separating gel was 7.5% acrylamide–0.18% bisacrylamide, pH 8.9, and the stacking gel, 0.125% acrylamide–0.03% bisacrylamide, pH 6.7. After the electrophoretic run, the gels were removed and stained for protein with Coomassie blue (27), for carbohydrate with basic fuchsin after periodate oxidation (28), and for lipid with oil red O (29).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The procedure followed was that described by Weber and Osborn (30). Densi-

tometric traces were recorded with a Gilford spectrophotometer equipped with a model 2410 linear transport accessory, scanning through a 0.1-mm slit at 550 nm for Coomassie blue, 560 nm for periodic acid–Schiff, and 510 nm for oil red O staining bands.

To locate the bound radioactivity, the gels were sliced with a Bio-Rad model 190 slicing device; 0.3 ml of "NCS" tissue solubilizer was added to individual slices, which then were left at room temperature with constant shaking overnight; 10 ml of Instabray were added, and the radioactivity was counted.

Reagents

[^{14}C]Dibenamine (6.8 mCi/mmole) was synthesized by Mallinckrodt Chemical Works. Nonradioactive dibenamine was a gift of Smith Kline & French Laboratories. N^1-Methylnicotinamide was obtained from Sigma Chemical Company; [^{14}C]NMN (3.6 mCi/mmole), from New England Nuclear Corporation; and all reagents for electrophoresis, from Bio-Rad. All other reagents were of the highest purity commercially available and were not further purified before use.

RESULTS

Tissue Slice Experiments

We have confirmed our earlier results (15) that dibenamine inhibits by 80–90% the transport of [^{14}C]NMN into renal cortical slices (eight experiments). In contrast, the irreversible inhibition of organic cation uptake by [^{14}C]dibenamine was decreased, i.e., "protected," by 13.7% ± 3.3% (n = 8) by 10 mM NMN. Another organic cation, tetrabutylammonium (10 mM), gave 15.7% ± 4.5% (n = 6) protection. NaCl (10 mM) afforded only 4% protection.

Osmotic Shock

Preliminary experiments showed that the extent of NMN protection was greater in the hypotonic $MgCl_2$ osmotic shock fluid than in the sucrose osmotic shock fluid, and therefore the former was the fraction selected for study. What protection was found in the hypertonic solution was present in the pellet (P_1) fraction. The percentage of protection found in S_2 and S_3 was 3.4% ± 1.9% (n = 11), while the pellet fraction (P_3) gave 12.4% ± 3.3% protection (n = 11) and therefore was used for the purification procedure.

Solubilization of Binding Protein

Pellet P_3 was solubilized as described under METHODS. In 11 experiments 45–62% of the membrane protein, along with the protective capacity, was released into the supernatant fraction. Preliminary experiments had shown that the presence of detergent reduced the protection by NMN as well as dibenamine binding. Therefore, extensive dialysis of the solubilized protein was required before assay. Other detergents, such as Triton X-100 and deoxycholate, were not useful; although more protein was released from the membrane(s), the protective phenomenon could not be observed, even after extensive dialysis. The specificity of the protective effect was examined, and the results (Table 1) showed that (a) high concentrations of NMN were required to produce the effect; (b) protection was not due to an ionic strength effect, since no significant protection was obtained with 10 mM NaCl; and (c) other cations that compete for transport, i.e., tetramethylammonium, tetraethylammonium, and tetrabutylammonium (5, 6, 8, 31), were effective, while *p*-aminohippuric acid was not. Dibenamine binding to BSA was measured in the presence and absence of NMN, and the percentage of protection (approximately 4%) was similar to that obtained for NaCl; therefore any protective effect less than 5% was considered to be nonspecific.

Isolation

The starting material was cortical slices from four kidneys having a total wet weight of 24 g. An osmotic shock cycle released 830 mg of protein; of this, 29% (240 mg) was recovered in pellet P_3. Solubilization of P_3 released 109 mg of protein, showing 19% protection with NMN.

After dialysis against 100 mM sodium

TABLE 1

Specificity of protective effect

NMN and the substituted ammonium ions were added as the chloride salts, and *p*-aminohippurate, as the sodium salt. Protection was calculated as described, from disintegrations per minute bound per milligram of protein from samples of equilibrium dialysis experiments. These results are the means of two experiments carried out with two different kidney preparations (triplicate determinations in each experiment).

Sample	Ligand	Ligand concentration	Protection
		mM	%
Soluble protein[a]	NMN	0.1	0
	NMN	1	3.6
	NMN	10	14.5
	Tetramethylammonium	10	9.3
	Tetraethylammonium	10	16.7
	Tetrabutylammonium	10	20.2
	NaCl	10	4.6
	p-Aminohippurate	10	2.8
BSA (0.1 mg/ml)	NMN	10	4.1
BSA (0.5 mg/ml)	NMN	10	2.2
BSA (1.0 mg/ml)	NMN	10	1.1
BSA (5.0 mg/ml)	NMN	10	3.1

[a] Detergent-solubilized protein (1.4 mg/ml), pellet P_3 (see Fig. 1).

phosphate buffer, pH 7.2, for 24 hr, the sample was chromatographed on DEAE-Sephadex A-50, and fractions were collected (Fig. 2). In all, five major protein peaks were recovered, one eluting with the column void volume. The over-all protein recovery was 81%.

The individual samples indicated by the bars in Fig. 2 were pooled into five fractions (I–V), concentrated, dialyzed, and assayed (Table 2). Dibenamine binding was found with each fraction, but only one (peak I) showed any significant protection by NMN. A problem encountered during the isolation is demonstrated by the data in Table 2: the sum of dibenamine binding to the fractions recovered (1.34×10^6 dpm) was greater than the total binding capacity of the original material (1.07×10^6 dpm; see line 1, Table 3), suggesting an increase in the availability of nonspecific binding sites.

The sample (peak I) was dialyzed against 27 mM Tris-glycine buffer, pH 8.3, and subjected to preparative disc electrophoresis (Fig. 3). The samples indicated by the bars were pooled into three fractions (A–C), dialyzed, and assayed as before. Only one peak (peak B) exhibited dibenamine binding (41,230 dpm/mg of protein)

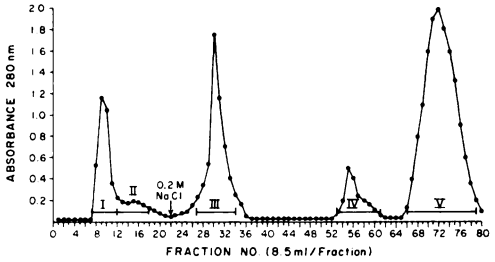

FIG. 2. *Anion-exchange chromatography*

DEAE-Sephadex A-50 was equilibrated with 100 mM sodium phosphate buffer, pH 7.2, and was used to prepare a column (2.5 × 40 cm) with a flow rate of 3.5 ml/cm²/hr. After the column had been charged with the sample, fractions were collected and their absorbance at 280 nm (●) was recorded. When the equivalent of 1 bed volume had been collected, 0.2 M NaCl was added to the buffer and additional fractions were collected. Fractions indicated by the bars were pooled, concentrated, dialyzed, and assayed for protection (see METHODS).

and protection (16,780 dpm/mg of protein in the presence of NMN, which amounts to 59%). Peak A did not bind dibenamine, while peak C bound 1210 and 1170 dpm/mg of protein in the absence and presence of NMN, respectively. Again, we recovered fractions displaying more than 100% of the total dibenamine binding capacity of the

originally applied material.

Peak B was dialyzed against 100 mM buffer, pH 7.2, for 24 hr and subjected to gel filtration on Agarose A-1.5 m. The protein elution pattern (85% recovery) (Fig. 4) gave two peaks: a small zone eluting with the void volume, and a broad peak. Fractions indicated by the bars were pooled and treated as above. Only the major peak (peak 2) showed dibenamine binding and protection (55%); 49,500 dpm of dibenamine were bound per milligram of protein in the absence of NMN, and 22,520 dpm/mg of protein, in its presence.

Table 3 summarizes the isolation procedure and shows a 15-fold purification after solubilization. The percentage of protection increased at each step, as expected if other proteins which bound dibenamine, but not NMN, were removed during the isolation. We interpret the protection as reflecting NMN binding, which can therefore be calculated from the difference in dibenamine binding. As shown in Table 3 (line 4), the purified protein bound 1.8 nmoles of NMN per milligram of protein. The final yield was 4% of the solubilized protein (0.3% of the osmotic shock fluid proteins), or 0.5 mg of purified binding protein per kidney.

TABLE 2

DEAE-Sephadex chromatography

Dibenamine binding was measured by equilibrium dialysis (see METHODS).

Fraction (peak)	Volume[a]	Protein	Dibenamine binding		Protection
			−NMN	+NMN	
	ml	*mg/ml*	*dpm/mg protein*		*%*
I	4.5	2.2	10,270	5,480	47
II	3.0	1.1	950	1,080	
III	7.5	3.8	18,680	18,240	2
IV	3.5	1.2	1,430	1,370	4
V	9.5	4.5	16,430	16,110	2

[a] Final volume of samples pooled as indicated by the bars in Fig. 1 after concentration with an Amicon ultrafiltration cell, using UM-10 membrane.

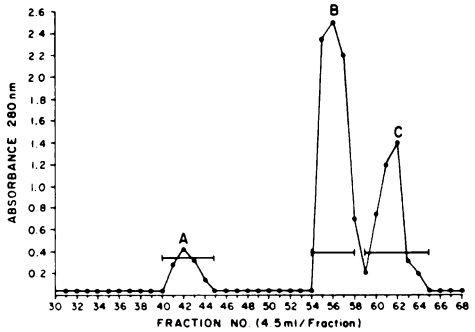

FIG. 3. *Preparative disc electrophoresis*

The fractions collected from a Canalco Prep-Disc instrument were monitored for absorbance at 280 nm (●). The fractions indicated by the bars were pooled, concentrated, dialyzed, and assayed for protection (see METHODS). The final volumes and protein concentrations were: peak A, 5.2 ml, 0.33 mg/ml; peak B, 6.0 ml, 0.50 mg/ml; peak C, 5.4 ml, 0.71 mg/ml. A 5% separating gel was prepared in a buffer of the same composition as the electrode buffer (27 mM Tris-glycine, pH 8.3). The elution buffer was 37.5 mM Tris-HCl, pH 7.4. A potential of 400 V was applied across the gel during the experiment, and the temperature was maintained at 4°.

TABLE 3

Purification of NMN binding-specific protein from dog kidney

Dibenamine binding was measured by equilibrium dialysis (see METHODS). NMN binding was calculated from NMN protection (change in dibenamine binding); [^{14}C]dibenamine had a specific activity of 6.8 mCi/mmole.

Fraction	Volume	Protein	Dibenamine binding		Protection	NMN binding	Protein yield
			−NMN	+NMN			
	ml	*mg/ml*	*dpm/mg protein*		*%*	*pmoles/mg protein*	*%*
1. Solubilized protein	26.0	4.2	9,870	8,030	19	120	100
2. DEAE-Sephadex	4.5[a]	2.2	10,270	5,480	47	320	11
3. Preparative disc electrophoresis	6.0[a]	0.5	41,230	16,780	59	1,620	7
4. Gel filtration	8.0[a]	0.27	49,500	22,520	55	1,790	4

[a] Volume after concentration with an Amicon ultrafiltration cell using a UM-10 membrane.

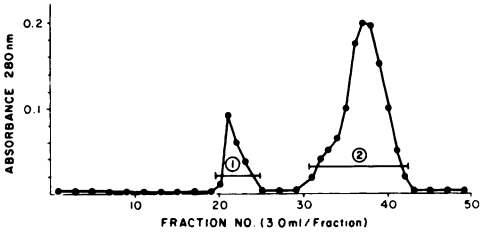

FIG. 4. *Gel filtration*

The gel filtration experiment was performed with Agarose A-1.5 m. After equilibration with 100 mM sodium phosphate buffer, pH 7.2, a column 1.0 × 100 cm was prepared with a flow rate of 51 ml/cm²/hr. Fractions were collected, and the absorbance at 280 nm (●) was measured. The fractions indicated by the bars were pooled, concentrated, dialyzed, and assayed for protection (see METHODS). The final volumes and protein concentrations were: peak 1, 4.0 ml, 0.11 mg/ml; peak 2, 8.0 ml, 0.27 mg/ml.

There was a substantial problem with stability during the isolation procedure. Although the protection capacity was uniformly demonstrated in 11 experiments, it was lost in eight other experiments at various steps during the isolation procedure. The reason for this instability is not known; it could not be prevented by the addition of glycerol, sulfhydryl reagents (2-mercaptoethanol and dithiothreitol), or detergent, or by variations in the solubilization procedure. To the extent that the experiments were successful, the results were reproducible: the protein peaks displaying protective capacity always appeared in the same relative positions, and in three experiments carried to completion the final yield was the same (0.5 mg of protein per kidney).

Homogeneity

Polyacrylamide gel electrophoresis patterns at various steps of the isolation procedure (Fig. 5A) illustrate the purification attained, and show a single protein band after the gel filtration step. Likewise, a concentration series (10–80 μg) of the purified protein (Fig. 5B) gave only a single protein-staining band, which also was positive for lipid and carbohydrate.

Addition of Lubrol to the electrophoretic system at a final concentration of 0.01–0.6% did not produce multiple bands, but did cause some spreading of the protein band (Fig. 5C).

The protein was analyzed further by means of SDS–polyacrylamide gel electrophoresis (Fig. 5D). Frame *1* shows the sol-

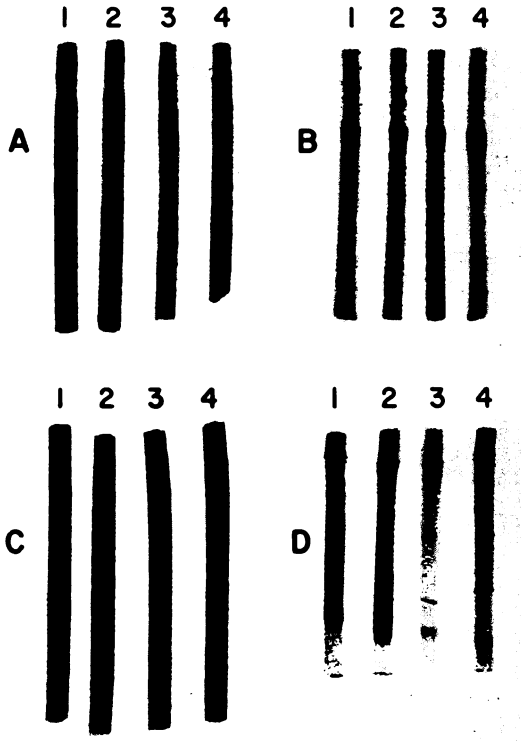

FIG. 5. *Tests of homogeneity*

Polyacrylamide gel electrophoresis experiments were performed after the method of Gabriel (25) to ascertain the state of purity of the binding protein. A. Gels at various stages of purification were stained for protein with Coomassie blue (27): *1*, solubilized pellet; *2*, after DEAE-Sephadex chromatography; *3*, after preparative disc electrophoresis; *4*, after gel filtration. B. Gels of the purified protein were stained with Coomassie blue (27) containing 10, 20, 40, and 80 μg of protein per gel (*1–4*, respectively). C. Polyacrylamide gels of the purified protein (5 μg) were stained for protein without (gel *1*) and with (gel *3*) 0.6% Lubrol WX added to the system. Control gels (no protein) with and without added detergent are *4* and *2*, respectively. D. SDS–polyacrylamide gels: *1*, solubilized pellet (P_3) material stained for protein (27); *2*, *3*, and *4*, purified protein stained for protein (27), carbohydrate (28), and lipid (29), respectively. The dark marks produced by injecting India ink into the gels indicate the position of the center of the tracking dye at the end of the electrophoretic run. The method was that described by Weber and Osborn (30), using 1% SDS and 1% 2-mercaptoethanol.

ubilized pellet (P_3) stained for protein, and frames *2*, *3*, and *4* represent the purified protein stained for protein, lipid, and carbohydrate, respectively. The dark mark, produced by injecting India ink into the gels, indicates the position of the center of the tracking dye at the end of the electrophoretic run. Analysis of the data shows that after treatment with SDS the protein was present as two protein-staining species. One migrated with an apparent molecular weight of less than 10,000 (based on calibration of the gel with several polypeptides of known molecular weight) and accounted for about one-third of the total mass (estimated from a densitometric trace); the second migrated faster than the tracking dye. In addition, separation of components after SDS treatment was indicated by zones positive for lipid (gel *3*) and for carbohydrate (gel *4*) that preceded the tracking dye.

The distribution of the components present in the rapidly migrating complex is best illustrated by examining the densitometric traces of the gels (Fig. 6A, B, and C). The carbohydrate-staining zone is the leading component, followed by the lipid- and then the protein-staining band. The lipid zone is not superimposable on the protein zone, but does overlap its leading edge.

If dibenamine acts as an active site-directed reagent, its position after SDS-gel electrophoresis should indicate the binding site(s). The samples from a protection experiment were subjected to SDS-gel electrophoresis, and the gels were sliced and counted (Fig. 6D). Counts per minute per slice were plotted against gel length (every other data point), assuming a uniform gel slice of 1 mm. The solid curve in Fig. 6D represents the radioactivity found in those gels containing protein alkylated by dibenamine (NMN absent), showing a narrow locus of significant binding (10 times background). In the presence of NMN (dashed curve) the dibenamine binding decreased just as expected if, indeed, the alkylation was at the active or NMN binding site. Inspection of the data also reveals that this locus does not coincide with the center of either protein-staining band. While this method of gel analysis is

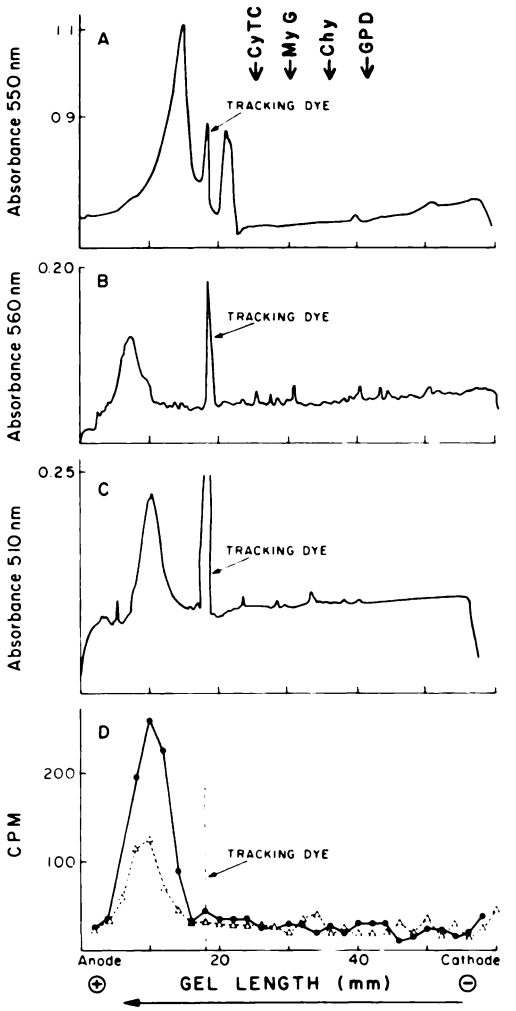

FIG. 6. *Distribution of components*

The SDS-polyacrylamide gels were analyzed by recording the absorbance at 550 nm for protein (A), at 560 nm for carbohydrate (B), and at 510 nm for lipid (C), using a Gilford spectrophotometer equipped with a model 2410 linear transport accessory, scanning through a 0.1-mm slit. To each gel, 17 μg of protein were applied. Localization of the covalently bound [^{14}C]dibenamine was determined by performing the protection assay (see METHODS), treating the sample with SDS (30), performing SDS-polyacrylamide gel electrophoresis, slicing the gel, and counting (D). The data (every other slice) are presented as disintegrations per minute per slice for samples containing [^{14}C]dibenamine (●——●) and [^{14}C]dibenamine plus 10 mM NMN (△- - -△). Molecular weight was estimated relative to the following standard proteins: cytochrome *c* (CyTC, 11,700), myoglobin (MyG, 17,000), chymotrypsinogen (Chy, 25,700), and glyceraldehyde 3-phosphate dehydrogenase (GPD, 36,000).

subject to limitations, the data suggest that a lipid moiety is fundamentally involved at the binding site.

The purified protein lost its capacity to be alkylated by dibenamine if it was first treated with SDS, whereas the protective capacity, but not dibenamine binding, was lost after thermal denaturation.

The organic cations listed in Table 1 which are known to be transported by this system (tetramethylammonium, tetraethylammonium, and tetrabutylammonium) displayed substantial protection at 10 mM against dibenamine binding to the purified protein (range, 57–67%), whereas *p*-aminohippurate was ineffective at this concentration. At lower concentrations of organic cations the percentage of protection was less (22% at 0.5 mM NMN), and reached a maximum (approximately 55%) at 50 mM, suggesting saturation.

DISCUSSION

We believe that we have isolated an organic cation-specific binding protein, based on the specificity found when using NMN to protect against dibenamine binding.

For the method of assay to be applicable, two conditions must be fulfilled: both the inhibition and the protection against alkylation must be specific. The first condition apparently is satisfied, since dibenamine blocks only the cellular uptake of organic cations, but not organic anion uptake or other functions (15). Ross *et al.* (15) showed that the inhibition was due only to the irreversibly bound form of dibenamine, not to a form that was taken up by the cell. The problem of nonspecific binding by an affinity reagent has been noted by other investigators (32–35), and our results show that dibenamine binds to many proteins, including BSA and various cytosol components (Tables 1 and 2). However, our results also show that the covalently bound dibenamine (Fig. 6D) is limited to a very definite locus, suggesting a high specificity for the binding site on that complex protein.

The second condition is the specificity of the protective effect. Other investigators (33–36) have reported on the lack of specificity of protection; that is, nonspecific as well as receptor sites were protected by the ligand against alkylation by dibenamine. Each of these studies was an attempt to characterize the *alpha* adrenergic receptor. Our results showed the organic cation protection to be highly specific: only organic cations gave protection (Table 1), and NMN protection was identified with a specific protein (Tables 2 and 3). The most likely interpretation is that the affinity reagent and the organic cations bind at the same site.

The finding that unphysiological concentrations of NMN were necessary for maximum protection exemplifies one of the difficulties in using an indirect binding assay for quantitation, in that saturation may in actuality limit dibenamine binding.

The binding protein appears to be an integral part of the membrane, since a detergent is required for its release (37). Once it had been solubilized, the inherent problems of the purification were complicated by the lack of stability, similar to other membrane proteins [for example, $(Na^+ + K^+)$-ATPase; see ref. 38]. We have not yet established the protocol for stablizing the binding protein, but the isolation was reproduced, although inconsistently.

The present results conflict with those reported by Magour *et al.* (23), in that we found the carrier-like protein in particulate fractions and not in the cytosol. Although both studies used dog kidney preparations, we used an equilibrium dialysis technique to measure protection, whereas Magour *et al.* (23) measured protection after precipitation with acid. We do not know whether nonspecific protection could be or was observed with their technique.

The isolated protein displays interesting properties: (a) it contains both lipid and carbohydrate; (b) the lipid moiety seems to be involved in dibenamine binding [similar observations have been made in studies on the *alpha* receptor (39)]; and (c) after SDS-gel electrophoresis the components are separated into a protein band (mol wt less than 10,000) and a complex containing protein, lipid, and carbohydrate, which migrates faster than the tracking dye. We suspect that this complex contains a hy-

drophobic protein which binds excess SDS and thus has an altered electrophoretic mobility (40–42). Preparative quantities of this component are necessary for a more thorough chemical description.

Although it is tempting to speculate that the isolated protein is the carrier for organic cations, the data are not yet sufficient to reach that conclusion. If further research supports the premise, the proposed model for transport will have to be altered, because the isolated protein contains carbohydrate, and the evidence is overwhelming that glycoproteins do not rotate through or move across membranes (37). Thus we shall have to postulate that the protein is more like an ionophore which perhaps spans the membrane, and through which the organic ions move.

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