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Covalent Modification of the Amine Transporter with *N,N'*-Dicyclohexylcarbodiimide†

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ABSTRACT: *N,N'*-Dicyclohexylcarbodiimide (DCC) has been previously shown to inhibit the amine transporter from chromaffin granules [Gasnier, B., Scherman, D., & Henry, J. P. (1985) *Biochemisty 24*, 3660-3667]. A study of the mechanism of inhibition is presented together with the demonstration of covalent modification of the protein. DCC inhibits binding of R1 (reserpine) and R2 (tetrabenazine) types of ligands to the transporter as well as transport. Ligands of the R2 type, but not those of the R1 type, protect against inhibition of all the reactions by DCC, i.e., accumulation of serotonin, binding if reserpine (R1 ligand), and binding of ketanserine (R2 ligand). The ability of a given R2 ligand to protect the transporter correlates well with its binding constant. Water-soluble carbodiimides, such as 1-ethyl-3-[3-(diethylamino)propyl]carbodiimide (EDC), do not have any effect on the catalytic activity of the transporter. A fluorescent hydrophobic analogue of DCC, *N*-cyclohexyl-N'-[4-(dimethylamino)-α-naphthyl]carbodiimide (NCD-4), inhibits at about the same concentration range as DCC. [^{14}C]DCC labels several polypeptides in the chromaffin granule membranes. Labeling of a polypeptide with an apparent M_r of 80K is inhibited in the presence of R2 ligands. The labeled polypeptide copurifies with the recently identified and isolated transporter [Stern-Bach, Y., Greenberg-Ofrath, N., Flechner, I., & Schuldiner, S. (1990) *J. Biol. Chem. 256*, 3961-3966].

The biogenic amine transporter is responsible for accumulation of serotonin, dopamine, norepinephrine, epinephrine, and histamine within secretory vesicles in a variety of cells (Kanner & Schuldiner, 1987; Njus et al., 1986). The energy required for amine accumulation comes from an ATP-driven H^+ pump in the secretory vesicle membrane which acidifies the vesicle lumen (Rudnick, 1986a,b). The amine transporter exchanges intravesicular H^+ ions for cytoplasmic biogenic amines, thereby coupling the downhill flux of accumulated H^+ ions with uphill amine accumulation.

Two distinct types of binding sites have been characterized: (a) Reserpine binds to the R1-type site, and its binding is inhibited by transport substrates (Deupree & Weaver, 1984; Scherman & Henry, 1984). (b) Tetrabenazine and ketanserine bind to the R2-type site, which has a low affinity for transport substrates (Scherman & Henry, 1983; Darchen et al., 1988).

Binding of [^{3}H]reserpine is accelerated upon imposition of a proton electrochemical gradient across the membrane (Scherman & Henry, 1984). Once bound, [^{3}H]reserpine dissociates very slowly, if at all, from the transporter (Rudnick et al., 1990). Binding is very stable, and reserpine remains bound even after solubilization, a finding that has proven very useful in the purification of the transporter (Stern-Bach et al., 1990).

We have designed a purification protocol, in conjunction with a functional reconstitution assay, which has succeeded to yield a highly purified, fully functional transporter. The transporter is a glycoprotein with an apparent M_r of 80K (Stern-Bach et al., 1990).

Carbodiimides have been used in several enzyme systems to demonstrate the involvement of carboxyls in the catalytic cycle (Cattell et al., 1970; Sussman & Slayman, 1983; Sachs et al., 1976; Pick & Racker, 1979; Webb & Taylor, 1987) and in some cases to identify the specific residues involved (Igarashi & Aronson, 1987; Cidon & Nelson, 1983).

The object of this paper is to test the involvement of carboxyls in the catalytic cycle of the amine transporter. In their initial study Henry and collaborators showed that *N,N'*-di-

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cyclohexylcarbodiimide (DCC)[1] inhibits transporter-mediated accumulation of amines. We tested whether inactivation is due to carboxyl modification as such or rather by internal cross-linking with neighboring molecules. In addition, we tested the interaction between the two ligand binding sites and the carbodiimide, and we probed the microenvironment of the carboxyl group. Finally, using [^{14}C]DCC we have been able to label a glycoprotein of apparent M_r 80K which copurifies with the amine transporter.

Experimental Procedures

Materials. [^{3}H]Reserpine was from the Nuclear Research Center, Negev, Bear Sheva, Israel; [^{3}H]ketanserin was from New England Nuclear; [^{3}H]serotonin and [^{14}C]DCC were purchased from Amersham. Ketanserin was a gift from Janssen (Janssen Chimica, Beerse, Belgium) and NCD-4 from Dr Uri Pick, Weizman Institute. All other reagents were of the highest quality commercially available.

Preparation of Chromaffin Granule Membrane Vesicles. Chromaffin granules were prepared from bovine adrenal glands essentially as described by Kirshner (1962). Membrane vesicles were obtained by osmotic shock, frozen, and stored in liquid air (Schuldiner et al., 1978).

Membrane Loading with K^+. Membranes (1 mL of a 5 mg of protein/mL suspension) were diluted into 40 mL of a prewarmed (37 °C) solution containing 10 mM K-Hepes (pH 7.4). After a 5-min incubation, 25 mM KSCN and 125 mM KCl were added and incubated for an additional 5 min at 37 °C. They were then centrifuged for 30 min at 40000*g* and resuspended in 10 mM K–Hepes, pH 7.4, 125 mM KCl, and 25 mM KSCN (KN) to the appropriate protein concentration.

ΔpH-Dependent Transport. ΔpH-dependent transport was assayed at 37 °C in 300 μL of a medium containing 0.3 M sucrose, 10 mM Tris–Hepes (pH 8.5), 2.5 mM $MgSO_4$, 5 μM nigericin, and 0.4 μM [^{3}H]serotonin (12.6 Ci/mmol). The reaction was initiated by a 100-fold dilution of membranes loaded with K^+ as described above (15–20 μg of protein). At a given time, uptake was terminated by rapid addition of 2 mL of a cold solution of the same buffer and immediate filtration through membrane filters (Schleicher and Schuell, 0.45-μm pore size). The filters were then washed with another 2 mL of the latter solution, removed from the suction apparatus, dried, and assayed for radioactivity by liquid scintillation spectrometry.

ATP-Dependent Transport. ATP-dependent transport of [^{3}H]serotonin was assayed essentially as previously described (Schuldiner et al., 1978).

Reserpine Binding. Membranes were diluted to a protein concentration of approximately 0.25 mg/mL in KN, pH 7.4. [^{3}H]Reserpine (20.1 Ci/mmol) was added to a final concentration of 3 nM, and the mixture was incubated for 4 h at 37 °C. At this time a 300-μL sample of the suspension is applied to a column of 2 mL of Sephadex G-50 fine which had been packed in a 2-mL disposable syringe and precentrifuged at 100*g* for 10 s. The column with applied sample was centrifuged once more for 1 min at 225*g* and the effluent counted in 4 mL of 40% Lumax (Lumac, Landgraaf, Netherlands) in toluene. Parallel reaction mixtures containing 2 μM reserpine were used to subtract nonspecific binding. The blank values were about 10% of the control.

Ketanserin Binding. Membranes were diluted to a protein concentration of approximately 0.25 mg/mL in 0.3 M sucrose containing 20 mM K-Hepes, pH 8.5, and 0.5 nM [^{3}H]-ketanserin (Gabay, 1990). The mixture was incubated for 5 min at 4 °C. Specific binding was measured essentially as described (Darchen et al., 1988): At the indicated times the reaction mixture was diluted with 2 mL of ice-cold SH, filtered on GF/C Whatman filters, and washed with an additional 2-mL aliquot. Parallel reaction mixtures containing 3 μM TBZ were used to subtract nonspecific binding. The blank values were about 20% of the control.

Purification of the Amine Transporter. Membranes were solubilized and fractionated essentially as described in Stern-Bach et al. (1990). Samples from different purification steps were precipitated with 4 volumes of a mixture of chloroform/methanol (1:3 v/v). The pellets obtained after centrifugation (34000*g*, 30 min) were dried under N_2 and resuspended in sample buffer (10% glycerol, 1% β-mercaptoethanol, 2% SDS, and 0.13 M Tris-HCl, pH 6.8). The samples were analyzed on 12.5% SDS–polyacrylamide gels as described (Laemmli, 1970). The gels were washed in water, incubated in 1 M sodium salicylate, and washed again in water prior to drying and exposure to film.

Results and Discussion

Inactivation by DCC Is Prevented by R2 Ligands Differentially. As previously shown by Gasnier et al. (1985), DCC inhibits not only the transporter-mediated accumulation of amines but also binding of two ligands of the transporter: reserpine and tetrabenazine. Since the latter two ligands have been proposed to bind to two distinct sites on the transporter, we tested whether both types of ligands have an effect on the rate of inactivation. We measured ΔpH-driven accumulation of serotonin, binding of reserpine (an R1 type of ligand), and binding of ketanserin (a novel R2 ligand): as expected, the three activities are inhibited to a similar degree (85%, 97%, and 90%, respectively) when membranes are incubated in the presence of 200 μM DCC (Figure 1, panels A, B, and C, respectively). Inhibition follows pseudo-first-order kinetics and is proportional to the DCC concentration in the range 20–200 μM (data not shown). When either tetrabenazine (0.1–3 μM, Figure 1) or ketanserin (5 μM, not shown) is added during preincubation with DCC, there is only a slight inhibition of the three activities as compared with controls incubated without DCC and in the presence of TBZ. However, when either serotonin (200 μM, Figure 1) or adrenalin (1 mM, not shown) was present during incubation with DCC, inactivation was as high as in their absence. Both compounds are substrates of the amine transporter which bind with high affinity to the R1 site but very poorly to the R2 site. Their failure to protect against DCC suggests that the reactive carboxyl group on the transporter is somehow associated with R2 site. Protection by the R2 ligands could be due to either a physical proximity to the R2 site or a conformational change imposed on the protein upon binding of R2, but not R1, ligands.

pH Dependence. In order to test the pH dependence of the rate of inactivation of transport, membranes were preequilibrated to pH 5.6, 7.4, or 8.5 in KN (titrated with KOH for pH 7.4 and 8.5 and with MES for pH 5.6) and then treated for 2 min with 100 μM DCC. Thereafter, they were collected, washed, and assayed for ΔpH-driven transport activity under identical conditions. The results show that inactivation is faster as pH is decreased. Membranes incubated at pH 8.5 were inactivated less than 5% under these conditions, while inactivation was 40% and 60% when the pH of the incubation was

[1] Abbreviations: KN, 10 mM K-Hepes, pH 7.4, 125 mM KCl, and 25 mM KSCN; DCC, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(diethylamino)propyl]carbodiimide; NCD-4, *N*-cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]carbodiimide; DES, diethylstilbestrol; SDS, sodium dodecyl sulfate; DEAE, (diethylaminoethyl)cellulose DE-52; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HTP, hydroxylapatite.

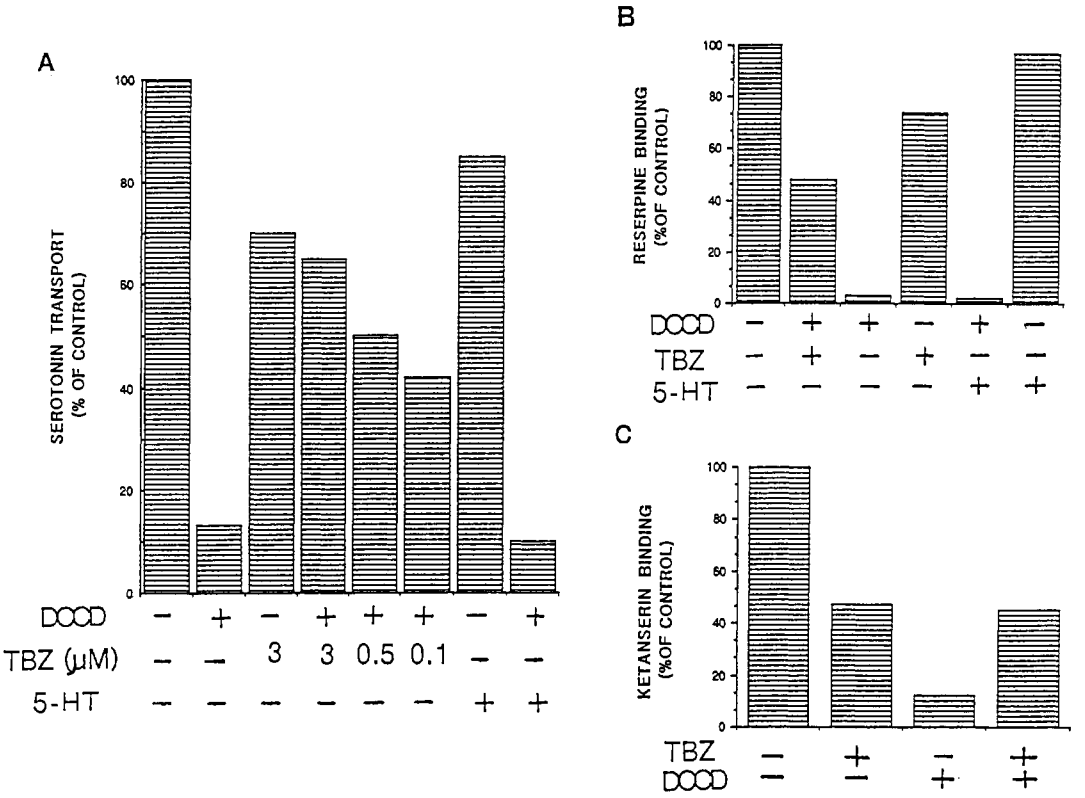

FIGURE 1: DCC inhibition of binding and transport. For measuring ketanserin (C) and reserpine (B) binding, membranes were preincubated in the presence or absence of 3 μM TBZ in 0.3 M sucrose and 10 mM K-Hepes, pH 7.4, for 20 min, followed by the addition of 200 μM DCC for an additional 20 min. The membranes were then washed three times by 10-fold dilution, centrifuged, and resuspended in the same buffer, pH 8.5 (0.25 mg of protein/mL). For measuring transport (A), membranes loaded with K^+ were preincubated with the indicated concentration of TBZ or serotonin during 20 min and then further incubated for 20 min with 200 μM DCC. After three washes and centrifugations they were resuspended in 10 mM K-Hepes, pH 7.4, 125 mM KCl, and 25 mM KSCN and assayed as described under Experimental Procedures. Control values were as follows: 197 pmol/(mg·40 s), 1.97 pmol/mg, and 0.33 pmol/mg, for uptake of serotonin (A), reserpine (B), and ketanserine (C) binding, respectively.

7.4 or 5.6, respectively.[2] For reaction with carbodiimides, carboxyl groups should be protonated (Khorana, 1953) and hence the optimal pH for inactivation probably reflects the carboxyl p*K*.

Carbodiimides can react with sulfhydryls and tyrosines in addition to carboxyls. The pH dependence of the reaction suggests that it is unlikely that DCC is reacting with either of the former groups unless they are unusually acidic ones. It is also sometimes possible to distinguish between these alternatives with externally added nucleophiles. Reaction of carboxyls with carbodiimides is a two-stage reaction involving activation of the carboxyl to an *O*-acylurea, and then rearrangement to a *N*-acylurea, or nucleophilic displacement by a neighboring or externally added nucleophile or hydrolysis (Carraway & Koshland, 1972). If the second stage of the carbodiimide reaction (rearrangement to the *N*-acylurea) is rate limiting, and hydrolysis to the free carboxylic acid occurs frequently, an externally added nucleophile should accelerate inactivation. Indeed, when 2 mM methylamine (Table I) was present during the treatment with DCC, the inactivation rate was consistently and reproducibly accelerated by 25%. The increased rate of inactivation at the pH decreased and the acceleration by nucleophiles is considered to support the involvement of a carboxylic acid (Shani-Sekler et al., 1988; Webb & Taylor, 1987; Carraway & Koshland, 1972).

Hydrophobicity of the Carboxyl Group. It is possible to probe the relative hydrophobicity of the microenvironment of the reactive carboxyl group by testing its reactivity toward carbodiimides with different structures. The results in Figure 2A show that EDC, a water-soluble carbodiimide, does not inhibit serotonin transport when it is driven by an artificially imposed pH gradient even at concentrations of up to 5 mM. On the other hand, when membranes are treated with the same EDC concentrations, ATP-driven transport is fully inhibited already at 2 mM EDC (Figure 2B). The latter reaction requires the activity of the H^+-ATPase, and results suggest therefore that a reactive carboxyl on the H^+ translocating

Table I: Stimulation of Transport Inhibition by Methylamine[a]

treatment	serotonin transport [pmol/(mg of protein·30 s)]			mean
control	472	497	399	456
	(14)	(25)	(20)	(20)
methylamine	512	480	402	464
	(35)	(24)	(20)	(26)
DCC	278	318	201	265
	(17)	(16)	(10)	(15)
DCC + methylamine	225	207	158	197
	(15)	(10)	(11)	(12)

[a] Membranes (1 mg of protein/mL) were loaded with K^+ as described under Experimental Procedures and treated with 200 μM DCC in the presence or absence of 2 mM methylamine at room temperature. After 1 min the membranes were diluted, centrifuged, and collected in ice-cold 0.3 M sucrose and 10 mM K-Hepes pH 7.4, as described in Figure 1. ΔpH-driven serotonin transport was assayed as described under Experimental Procesures. The values in the table indicate the results of three independent experiments. The standard deviations are indicated in parentheses.

[2] Control values for membranes incubated without DCC were 315, 368, and 260 pmol/(mg·40 s) for membranes at pH 5.6, 7.4, and 8.5, respectively.

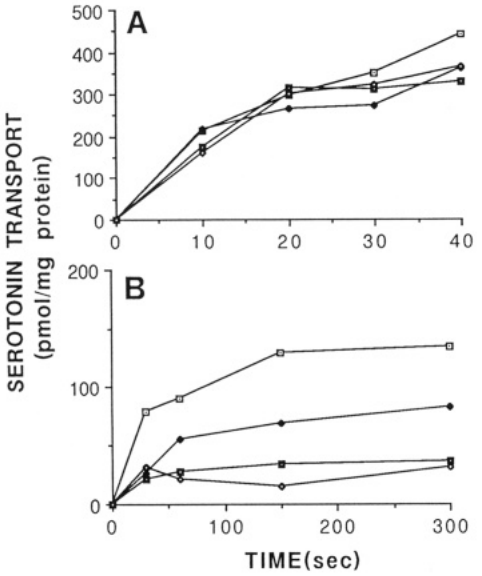

FIGURE 2: Effect of EDC on ATP-dependent and ΔpH-induced serotonin transport. Membranes (1 mg of protein/mL) were loaded with K^+ before treatment as described under Experimental Procedures. After suspension in KN at 1 mg of protein/mL they were incubated with EDC during 20 min at room temperature at the following concentrations: (□) 0, (◆) 0.5, (■) 2.0, and (◇) 5.0 mM. After washing, either ATP-dependent (B) or ΔpH-driven (A) [^{3}H]serotonin uptake was assayed.

ATPase is more exposed to the hydrophylic carbodiimide EDC than the carboxyl on the amine transporter.

A novel fluorescent carbodiimide, NCD-4, has been reported to inhibit the sarcoplasmic reticulum Ca-ATPase (Chadwick & Thomas, 1983; Pick & Weiss, 1985). NCD-4 incorporated into the latter enzyme, resulting in a fluorescent *N*-acetylurea derivative. The hydrophobicity of this derivative is very similar to that of DCC. When the effect of NCD-4 on the transporter is tested, transport is inhibited at concentrations very similar to those used with DCC (50% inactivation is obtained when membranes are treated with 50 μM NCD-4 at room temperature for 20 min).

We can conclude from the above that the reactive carboxyl on the amine transporter is relatively hydrophobic since EDC, a water-soluble carbodiimide, has no effect on the transporter. However, the site does not seem to be completely occluded from a hydrophilic environment since hydrophilic nucleophiles and changes in the concentration of hydrogen ions in the medium do have an effect on the rate of inactivation.

Labeling of an 80-kDa Glycoprotein with [^{14}C]DCC. The formation of stable protein–DCC adducts can be followed by labeling DCC with a radioactive isotope. When chromaffin granule membranes are labeled with [^{14}C]DCC and fluorographed after SDS–PAGE analysis, several polypeptides are labeled irrespective of the presence of protective ligands (not shown, but see Figure 4, lane 1, to estimate the complexity of labeling). This result is not surprising since the fraction of transporter is estimated to be only about 0.02% of the membrane proteins (Stern-Bach et al., 1990) and its labeling was not necessarily visible under the separation conditions shown. The protection of ligands against labeling with DCC can be unveiled, however, upon enrichment of the amine transporter by about 50–100-fold. Thus membranes treated with [^{14}C]DCC in the presence or absence of 25 μM TBZ were solubilized, and the extract was fractionated on a DEAE column as previously described (Stern-Bach et al., 1990). Analysis of the fraction enriched with the amine transporter reveals a much simpler labeling pattern: Two major (60 and 100 kDa) and two minor bands (45 and 80 kDa) are detected;

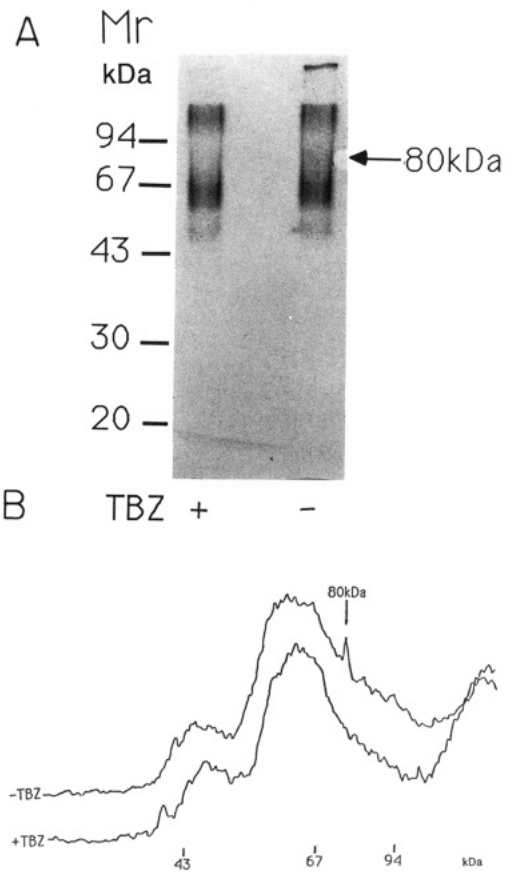

FIGURE 3: Covalent modification of membrane proteins with [^{14}C]DCC and specific protection by TBZ. (A) Membrane vesicles (1 mg of protein/mL) suspended in 4 mL of SH were treated with 100 μCi of [^{14}C]DCC for 20 min at pH 7.0 in the absence (right) or in the presence of 25 μM TBZ (left). Triton X-100 (1.5%) was then added, and the suspension was centrifuged at 100000*g* for 60 min. The supernatant was chromatographed on a DE-52 column as described (Stern-Bach et al., 1990). The fraction eluted at 150 mM NaCl is highly enriched with the transporter and contains only 2% of the original protein, resulting in about 50–100-fold purification. The samples were concentrated and analyzed on a 12.5% SDS–polyacrylamide gel. The gel was fluorographed as described under Experimental Procedures. Molecular weight of standard proteins is shown on the left. (B) The fluorograph was scanned with a densitometer from Quick Scan Helena Laboratories. Molecular weights of markers are shown at the bottom.

the intensity of only one of them is diminished significantly when labeling was done in the presence of TBZ (Figure 3). The protected peptide shows a diffuse pattern corresponding to an apparent M_r of 80 000. This pattern is similar to that of the purified amine transporter. The 80-kDa polypeptide can be further purified on a hydroxylapatite column as described for the amine transporter. A single band is detected on the autoradiogram of the fraction containing the transporter (Figure 4). The apparent M_r of the [^{14}C]DCC-labeled band is identical with that of the transporter.

We conclude that DCC forms a stable adduct with a carboxyl group on the amine transporter. The properties displayed by this carboxyl suggest a direct relation, either physical or functional, with the R2 site on the protein. In addition, our results suggest a certain hydrophobic environment in the vicinity of this carboxyl that makes it available to DCC and NCD-4 but not EDC. In addition, our data suggest that the site is available to small nucleophiles and to hydrogen ions as well.

The demonstration of the formation of a stable adduct supports the involvement of a carboxyl group in the catalytic cycle. Its identification should be of value in our endeavor

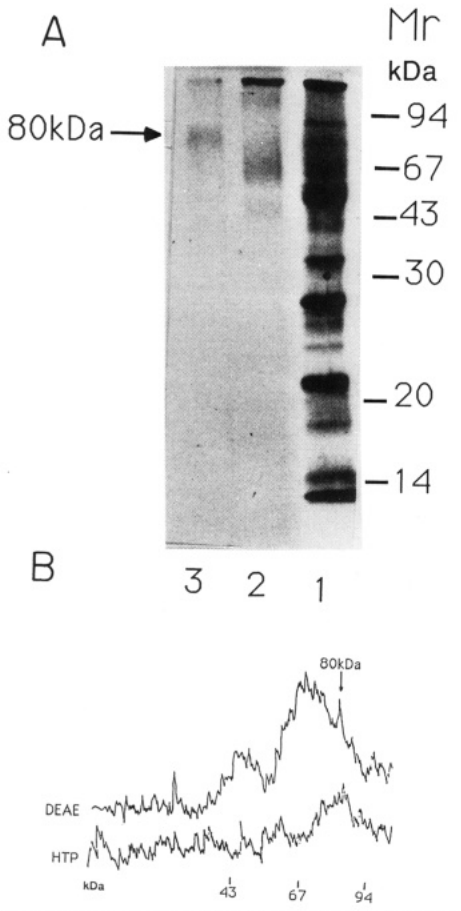

FIGURE 4: The DCC-labeled 80-kDa polypeptide copurifies with the amine transporter. Membrane vesicles (2 mg) were labeled with 40 μCi of [^{14}C]DCC solubilized and chromatographed in a DE-52 column and analyzed as described in Figure 3. The high-salt fraction was further chromatographed on an HTP column (Stern-Bach et al., 1990). The amounts of the total protein (in μg) and the amine transporter (in pmol) loaded in each lane are indicated in parentheses. The amounts of the amine transporter loaded were calculated according to Table II in Stern-Bach et al. (1990). (A) Lane 1: Void volume of the DEAE column (6 μg of protein, 0.01 pmol). Lane 2: High-salt (150 mM NaCl) fraction of the DEAE column (1 μg of protein, 1.7 pmol). Lane 3: KP_i fraction (170 mM) of an HTP column which is highly enriched with the transporter (Stern-Bach et al., 1990) (1 μg of protein, 4.2 pmol). (B) Densitometric scan of lanes 2 and 3.

to understand the structure and the function of the protein.

Registry No. 5′-ATP, 56-65-5; serotonin, 50-67-9.

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