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# Proline Transport by Synaptosomal Membrane Vesicles Isolated from Rat Brain: Energetics and Inhibition by Free Fatty Acids†

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ABSTRACT: Synaptosomal membrane vesicles have been employed to study the energetics of proline transport and the inhibition of proline transport by unsaturated free fatty acids. Active uptake of proline into synaptosomal membrane vesicles requires extravesicular $Na^+$ and is primarily driven by a $Na^+$ gradient created by diluting $K^+$-loaded vesicles into $Na^+$-containing buffers. Uptake of proline under these conditions is enhanced up to 2-fold by a valinomycin-induced diffusion potential (interior negative). Proline transport is reduced in the absence of external $Cl^-$ or internal $K^+$. Strong (40–90%) inhibition of proline uptake occurs upon collapse of the $Na^+$ gradient by ionophores such as gramicidin D or activation of the action potential $Na^+$ channel by veratridine or *Tityus serrulatus* venom. Less (15–25%) inhibition is obtained with the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, which also prevents the stimulation of proline uptake by the valinomycin-induced diffusion potential. Unsaturated free fatty acids inhibit proline uptake. The inhibition is greatest for arachidonic acid and was somewhat less for oleic acid. The saturated fatty acids palmitic and stearic have little or no inhibitory capacity. Endogenous unsaturated free fatty acids may exert similar inhibitory effects on the reuptake systems for neuroactive amino acids and thus modulate their action in the central nervous system.

High-affinity, sodium-dependent uptake systems for proline and other putative neurotransmitters have been described in isolated nerve ending (synaptosome) preparations (Peterson & Raghupathy, 1972; Bennett et al., 1973; Snyder et al., 1973). It has been suggested that such transport systems function as reuptake mechanisms in the termination of neurotransmitter actions (Iversen, 1971). Alternatively, since amino acid neurotransmitters are localized primarily in the cytoplasm rather than in synaptic vesicles, these transport systems may operate in the reverse direction under depolarizing conditions and release neurotransmitters from the nerve ending (O'Fallon et al., 1981). The inhibition of proline uptake by

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the ($Na^+$,$K^+$)-ATPase[1] inhibitor ouabain and by mitochondrial uncouplers (Rhoads et al., 1982a) suggested that this uptake is a secondary active transport process that is driven by ion gradient(s) created by the ($Na^+$,$K^+$)-ATPase and dependent on a mitochondrial supply of ATP. The sensitivity of proline transport to veratridine (Rhoads et al., 1982a) and to *Tityus serrulatus* venom (Rhoads et al., 1982b), both of which activate the action potential $Na^+$ channel, indicated an association of proline transport with the electrochemical potential of $Na^+$ and provided evidence that proline accumulation was largely associated with the presynaptic nerve terminals. The uptake of proline and other $Na^+$-dependent transport processes have also been shown to be inhibited specifically by relatively low concentrations of unsaturated free fatty acids (Rhoads et al., 1982c). This finding, together with the earlier demonstration that the uptake of amino acids by high-affinity, $Na^+$-dependent transport systems was stimulated by bovine serum albumin (Raghupathy et al., 1978; Peterson et al., 1979; Rhoads et al., 1982a), had led to the suggestion that the activity of these systems may be modulated in vivo by endogenous free fatty acids (Rhoads et al., 1982c).

Membrane vesicles, prepared from a variety of mammalian [e.g., Garcia et al. (1980a,b) and Sacktor et al. (1981)] and bacterial (MacDonald et al., 1977; Goto & Mizushima, 1978) cells, have been shown to be extremely useful for the study of amino acid transport, particularly in relation to the hypothesis that solute accumulation can be achieved by coupling to electrochemical gradients of ions. Membranes, isolated after hypoosmotic lysis of synaptosomes, reseal to form vesicles that accumulate a number of established and putative neurotransmitters (Kanner, 1978, 1980; Kanner & Sharon, 1978; Lahmesmaki et al., 1977; Campbell, 1978; Roskoski, 1981). These vesicles lack the complex energetics, metabolism, and compartmentation of intact synaptosomes and thus provide a model system for studying transport. The present study describes the energetics of the active transport of L-proline by a membrane vesicle preparation obtained after osmotic lysis of rat brain synaptosomes. Concentrative uptake took place in the absence of any exogenous energy source other than that imposed by artifically created ion gradients. Transport under these conditions was modified by ionophores and compounds that activate the voltage-sensitive $Na^+$ channel. Unsaturated free fatty acids inhibit vesicular proline transport by a mechanism that is independent of the source of energy that drives the transport. The data are consistent with the notion that this inhibitory action results from a direct effect on the proline carrier or on the lipid domain surrounding the carrier.

## Experimental Procedures

### *Materials*

L-[U-$^{14}$C]Proline (293 mCi/mmol), [*carbonyl*-$^{14}$C]inulin (2.4 mCi/g) and [$^3$H]$H_2O$ (1 mCi/g) were purchased from New England Nuclear Corp. (Boston, MA). Valinomycin, veratridine, gramicidin D, palmitic acid, stearic acid, oleic acid, arachidonic acid, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ouabain, and *T. serrulatus* venom were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrodotoxin (TTX) was obtained from Calbiochem (La Jolla, CA). Bovine serum albumin (fraction V, fatty acid free) was purchased from Miles Laboratories, Inc. (Elkhart, IN).

### *Methods*

*Preparation of Membrane Vesicles.* Synaptosomal fractions were prepared from brain cortices of adult Sprague-Dawley rats by the method of Kurokawa et al. (1965). A membrane fraction was obtained by osmotic rupturing of the isolated synaptosomes in a hypotonic buffer as described by Kanner (1978). Membranes were further purified from this lysed fraction by combined floatation–sedimentation density gradient centrifugation (Jones & Matus, 1974). The isolated membranes were suspended in 0.32 M sucrose containing 1 mM $MgSO_4$, 0.5 mM EDTA, and 5 mM Tris–sulfate, pH 7.4, at a protein concentration of 10–15 mg/mL. Protein was measured by the method of Lowry et al. (1951). Membrane suspensions were stored frozen and used within 1–2 weeks of their preparation.

*Transport Assays.* Membrane vesicles were loaded with buffer of the desired ionic composition by rapid thawing during dilution to 2–3 mg/mL with a loading solution at 37 °C (Rudnick, 1977). The composition of the loading solution was varied as described in the text and tables; the standard loading solution (TMK) consisted of 150 mM KCl, 1 mM $MgSO_4$, and 5 mM Tris–sulfate, pH 7.4. Of the diluted vesicle suspension, 50–100 μL was added to 0.9 mL of an incubation solution (TMNa) consisting of 150 mM NaCl, 1 mM $MgSO_4$, and 5 mM Tris–sulfate, pH 7.4, with 0.35 μM (0.1 μCi) uniformly $^{14}$C-labeled L-proline. Variations in the composition of the incubation solutions are described in the tables. Incubations were carried out in duplicate. Fatty acids were added at the required concentration in ethanol (10 μL). Control incubations received equivalent amounts of ethanol. Ethanol, at the concentrations used, had no effect on the parameters studied. After incubations for various times at 25 °C, reactions were terminated by the addition of 5 mL of ice-cold TMNa. Vesicles were collected by filtration on Millipore membrane filters (0.8 μm) and washed with 15 mL of buffer. The complete collection and wash procedure took approximately 15 s. The washed filters were placed in a toluene-based scintillation mixture, and the radioactivity was determined by liquid scintillation spectrometry. Values obtained in experimental incubations were expressed as a percent of the control values and are presented as the means ± standard deviations of independent values obtained from three or four different vesicle preparations. The amount of radioactivity associated with the filters was decreased by about 90% by washing with distilled water instead of buffer. This decrease was indicative of uptake into an osmotically sensitive compartment. All values were corrected for gradient-specific uptake by subtracting the amount of activity associated with vesicles incubated with [$^{14}$C]proline in the loading solution or by loading vesicles with incubation solution. In neither of these cases were ion gradients imposed on the system. The amounts subtracted represented 10–15% of the total radioactivity on the filters. In contrast to the gradient-specific uptake, these values were not osmotically sensitive nor ion- or temperature-dependent and were thus assumed to represent binding rather than uptake.

*Total Internal Volume.* The vesicular volume was determined by the method of Padan & Rottenberg (1973). [$^{14}$C]Inulin was used to measure the external osmotic space and [$^3$H]$H_2O$ was used to determine the total water. The volume was also determined by the method of Mehlhorn et

[1] Abbreviations: ATPase, adenosinetriphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TMK, solution consisting of 150 mM KCl, 1 mM $MgSO_4$, and 5 mM Tris–sulfate, pH 7.4; TMNa, solution consisting of 150 mM NaCl, 1 mM $MgSO_4$, and 5 mM Tris–sulfate, pH 7.4; Tempone, 2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxy; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TTX, tetrodotoxin; FFA, free fatty acids; GABA, γ-aminobutyric acid; Δ[$Na^+$], the initial concentration difference for NaCl between the intravesicular and extravesicular compartments; BSA, bovine serum albumin.

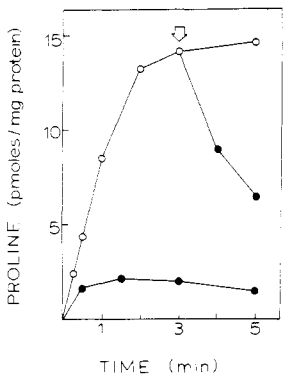


FIGURE 1: Proline uptake by synaptosomal membrane vesicles (○). Vesicles were loaded with TMK and resuspended in TMNa as described under Experimental Procedures. Gramicidin D was added at a final concentration of 10 μM, either at the start of the incubation (●) or after proline accumulation had reached a maximum [(●) after the arrow].

al. (1982) by employing electron spin resonance. The membrane-permeable spin probe 2,2,6,6-tetramethyl-4-oxopiperidinyl-1-oxy (Tempone) was employed, and the signal arising from extravesicular Tempone was quenched by the addition of the impermeable transition metal complex nickel–tetraethylenepentamine to observe only the intravesicular signal. This final signal is expressed as a fraction of the initial (total) signal, which is then related to the fraction of the total volume of the sample.

*Membrane Electrical Potential* ($\Delta\Psi$). The presence of an electrical potential (interior negative) was established by the use of spin-labeled phosphonium ions and techniques of external signal quenching similar to those described for the volume measurements (Mehlhorn et al., 1981). In this case, the large phosphonium ion, whose positive charge center is surrounded by hydrophobic groups, responds to transmembrane electrical potentials by being accumulated within the more negative internal aqueous compartment.

## Results

*Vesicular Volume and Electrical Potential.* The intravesicular volume was 1.5 μL/mg protein as determined by the electron spin resonance technique and 4.5 μL/mg protein by the [$^{14}$C]inulin/[$^{3}$H]$H_2O$ method. The smaller value obtained by the spin resonance technique may be expected since the addition of the metal ion complex to quench the signal arising from the extravesicular probe increased the osmolarity of the extravesicular buffer.

An intravesicular signal from vesicles loaded with TMK and resuspended in TMNa was obtained with the spin-labeled phosphonium ions. Such a signal is indicative of an electrical potential (interior negative), $\Delta\Psi$. The average line heights of this signal were decreased by 25–50% by the addition of extravesicular KCl. This reduction was consistent with the presence of a $K^+$ diffusion potential. The line heights were 30–50% greater in the presence of 5 μM valinomycin. The increased $K^+$ permeability due to valinomycin therefore resulted in a stimulated membrane potential ($\Delta\Psi_v$).

*Characteristics of Proline Transport.* Vesicles, loaded with TMK and diluted into TMNa, took up proline with a time course illustrated by Figure 1. This transport of proline was dependent on the artifically imposed ion gradients as was shown by the effects of the ionophore gramicidin D. Gramicidin D decreased both the initial rate of uptake and the overall accumulation of proline. In addition, when gramicidin D was added to vesicles that had accumulated proline to their maximum capacity (indicated by the arrow in Figure 1), a high efflux of proline ensued. The amount of proline accumulated ranged in different preparations from 10 to 30 pmol/mg of protein. This basal uptake of proline from TMNa- into TMK-loaded vesicles showed saturation kinetics. As determined from double-reciprocal plots, the $K_m$ for proline was 4.6 ± 1.4 μM ($N$ = 3), and the $V_{max}$ was 162 ± 52 pmol min$^{-1}$ (mg of protein)$^{-1}$ ($N$ = 3). The activation of proline transport by $Na^+$ was of second order with a Hill coefficient of 2.2 ± 0.4 ($N$ = 3).

Table I: Requirement for Sodium and Anion Specificity for Vesicular Proline Transport

| incubation solution[a] | proline accumulation (% of control) |
|---|---|
| 150 mM NaCl (control) | 100 |
| 150 mM $NaNO_3$ | 61 ± 9 |
| 100 mM sodium phosphate buffer, pH 7.4 | 38 ± 7 |
| 150 mM LiCl | 8 ± 5 |
| 150 mM KCl | 2 ± 1 |
| 150 mM Tris-HCl | 2 ± 1 |

[a] The incubation solution consisted of 5 mM Tris-sulfate, pH 7.4, 1 mM $MgSO_4$, and the salt indicated, with 0.35 μM $^{14}$C-labeled proline (0.1 μCi). The loading solution in each case was 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM $MgSO_4$.

Table II: Role of a Potassium Diffusion Potential in Vesicular Transport of Proline

| incubation solution[a] | proline accumulation (% of control) |
|---|---|
| 150 mM NaCl (control) | 100 |
| 150 mM NaCl + 5 μM valinomycin | 165 ± 10 |
| 150 mM Tris-HCl | 2 ± 1 |
| 150 mM Tris-HCl + 5 μM valinomycin | 2 ± 1 |
| 120 mM Tris-HCl + 30 mM NaCl | 10 ± 5 |
| 120 mM Tris-HCl + 30 mM NaCl + 5 μM valinomycin | 28 ± 9 |

[a] The incubation solution consisted of 5 mM Tris-sulfate, pH 7.4, 1 mM $MgSO_4$, and the salts indicated with 0.35 μM $^{14}$C-labeled proline (0.1 μCi). The loading solution in each case consisted of 5 mM Tris-sulfate, pH 7.4, 1 mM $MgSO_4$, 120 mM KCl, and 30 mM NaCl.

The replacement of chloride ion in the loading solution with orthophosphate ion, achieved by substituting potassium phosphate buffer (pH 7.4) for KCl, had no effect on proline uptake (data not shown). Substitution of Tris-HCl for KCl, however, resulted in uptake that was reduced by 10–20%. Replacement of KCl with NaCl or sodium phosphate resulted in complete loss of proline uptake. Uptake of proline was reduced in the presence of external anions other than $Cl^-$ (Table I). The extent of transport followed the order of the relative membrane permeability of the anions: $Cl^- > NO_3^- > PO_4^{3-}$. Substitution of LiCl, KCl, or Tris-HCl for NaCl resulted in little or no proline uptake. Such substitutions eliminate the $Na^+$ gradient while maintaining the $Cl^-$ gradient and/or $K^+$ gradient. Addition of sodium phosphate to both the loading and incubation solutions to eliminate the sodium gradient (data not shown) had no effect on the inability of the $Cl^-$ gradient and/or $K^+$ gradient to drive transport. These results demonstrate a strict dependence on $Na^+$ since neither the $K^+$ nor $Cl^-$ gradient alone could energize transport.

The effect of $\Delta\Psi_v$ was examined (1) in the presence of an initial $Na^+$ gradient ($\Delta[Na^+]$), (2) in the presence of $Na^+$ (but not $\Delta[Na^+]$), and (3) in the near absence of $Na^+$ (Table II). In the absence of $Na^+$, no transport occurred, and this was not altered by valinomycin. With 150 mM NaCl in the in-

Table III: Effects of Various Inhibitors on Sodium-Dependent Transport of L-Proline

| inhibitor ($\mu$M)[a] | proline accumulation (% of control) |
|---|---|
| ouabain (200) | 104 ± 1 |
| gramicidin D (10) | 17 ± 4 |
| CCCP (10) | 78 ± 9 |
| valinomycin (10) | 153 ± 13 |
| veratridine (25) | 60 ± 6 |
| veratridine (25) + TTX (5) | 93 ± 5 |
| TTX (5) | 106 ± 5 |
| *T. serrulatus* venom (5 $\mu$g/mL) | 66 ± 12 |
| *T. serrulatus* venom (5) + TTX (5) | 90 ± 6 |

[a] Inhibitors were added in the final concentrations shown to the incubation mixture that otherwise contained 150 mM NaCl, 1 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.4, and 0.35 $\mu$M [$^{14}$C]proline. The synaptosomal membrane vesicles were loaded with 150 mM KCl, 1 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.4, and were added to the incubation mixture at 0.15–0.3 mg/mL. Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TTX, tetrodotoxin.

cubation solution, i.e., with a $\Delta[Na^+]$, valinomycin stimulated transport to a level that was over 1.5 times the control value. In the presence of $Na^+$, but with equal concentrations of $Na^+$ in the loading and incubation solutions, a small amount of uptake occurred that was stimulated by valinomycin. Even this stimulated accumulation, however, was less than a third of that occurring under control conditions. Valinomycin had no effect on transport in the absence of $K^+$ gradient (in > out). Since valinomycin increased the magnitude of $\Delta\Psi$ under basal conditions, its stimulation of transport can be related to the increased $K^+$ diffusion potential, $\Delta\Psi_v$. The proton ionophore CCCP, added in the presence of valinomycin, completely prevented the stimulation of proline uptake by valinomycin.

While proline transport was sensitive to ionophores such as gramicidin D and valinomycin, it was not affected by the ($Na^+$,$K^+$)-ATPase inhibitor ouabain (Table III). This finding supports the contention that the artificial ion gradients are the sole source of energy driving proline transport. The proton ionophore CCCP inhibited slightly the basal uptake, suggesting that the small $\Delta\Psi$ measured under the basal conditions was contributing to the uptake of proline but that the major driving force for uptake under these conditions was $\Delta[Na^+]$ and thus CCCP independent. Veratridine and *T. serrulatus* venom inhibited the basal uptake of proline (Table III). That $Na^+$ channels are involved in this inhibitory effect was demonstrated in each case by reversal of the inhibition by tetrodotoxin. Tetrodotoxin alone had no effect on transport. The CCCP-independent uptake of proline was also inhibited by both veratridine and *T. serrulatus* venom (data not shown), indicating that while the increased $Na^+$ permeability resulting from their action may dissipate $\Delta\Psi$, their effect on $\Delta[Na^+]$ was critical to their inhibition of the basal uptake of proline.

The results in Figure 2 demonstrate the delineation of three energetic states for vesicular proline transport that can be contrasted to the basal uptake. The first, which occurred in the presence of $\Delta[Na^+]$ and $\Delta\Psi_v$, represented the maximum initial rate and overall accumulation of proline. The second occurred under conditions where $\Delta\Psi$ (or $\Delta\Psi_v$) was neutralized by the counter movement of protons catalyzed by CCCP, leaving only $\Delta[Na^+]$ to drive transport. The uptake under this energetic state ($\Delta[Na^+]$, $\Delta\Psi = 0$) was from 68 to 78% of that occurring under the basal conditions ($\Delta[Na^+]$, $\Delta\Psi$). The third state was established by a potassium diffusion potential, $\Delta\Psi_v$ in the absence of $\Delta[Na^+]$. In this case the loading and incubation solutions contained identical sodium concentrations

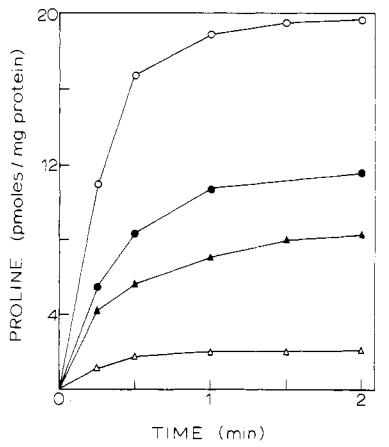


FIGURE 2: Proline uptake into membrane vesicles under different energy states: (I) sodium gradient and a valinomycin (5 $\mu$M)-induced membrane potential (○); (II) sodium gradient alone, with membrane potential abolished by 10 $\mu$M CCCP (▲); (III) a valinomycin-induced membrane potential alone, with sodium present but not as a gradient (△); and basal uptake (●). In each case the vesicles were loaded with 5 mM Tris–sulfate, pH 7.4, 1 mM $MgSO_4$, 120 mM KCl, and 30 mM NaCl. The incubation solution consisted of 5 mM Tris–sulfate, pH 7.4, 1 mM $MgSO_4$, and 150 mM NaCl, except in (III) where the 150 mM NaCl was replaced with 120 mM Tris-HCl, 30 mM NaCl.

Table IV: Effects of Free Fatty Acids and Bovine Serum Albumin on Vesicular Proline Transport[a]

| | proline accumulation (% of control) |
|---|---|
| saturated free fatty acids | |
| palmitic | 90 ± 8 (10) |
| stearic | 104 ± 15 (6) |
| unsaturated free fatty acids | |
| oleic | 39 ± 9 (6) |
| arachidonic | 19 ± 6 (6) |
| bovine serum albumin (BSA) | 151 ± 22 (6) |
| BSA + oleic acid | 163 ± 10 (3) |

[a] Free fatty acids (25 $\mu$M) or bovine serum albumin (15 $\mu$M) was added to the incubation mixture that otherwise consisted of 150 mM NaCl, 1 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.4, and 0.35 $\mu$M [$^{14}$C]proline. Vesicles, loaded with 150 mM KCl, 1 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.4, were added to a final concentration of 0.1 mg/mL.

as described in Table II. No uptake occurred in the total absence of sodium or if a gradient of potassium ($[K^+]$ intravesicular > $[K^+]$ extravesicular) had not been established prior to the addition of valinomycin.

*Inhibition by Free Fatty Acids.* The effects of free fatty acids on the basal proline uptake are shown in Table IV. The saturated fatty acids palmitic and stearic had little or no effect at a concentration of 25 $\mu$M on proline transport. The unsaturated fatty acids oleic and arachidonic strongly inhibited proline transport. Bovine serum albumin stimulated the basal uptake of proline and overcame the inhibitory effects of unsaturated free fatty acids.

## Discussion

Membrane vesicles were prepared from synaptosomal fractions and, most likely, are derived from the synaptosomal plasma membrane. A major portion of the proline transport system is located in compartments also containing veratridine-sensitive sodium channels, which, like the veratridine-sensitive transport systems of other neuroactive amino acids (Kanner, 1980), is evidence of their association with the presynaptic membrane. Vesicles prepared by similar methods

have met morphological and enzymatic criteria for presynaptic membranes (Jones & Matus, 1974). Potassium-loaded vesicles incubated in a potassium-free buffer develop a potassium diffusion potential (inside negative), which is enhanced by valinomycin as shown by the electron spin resonance of accumulated spin-labeled phosphonium ions. These vesicles are able to maintain artificially established sodium gradients as shown by their ability for concentrative accumulation of proline and its abolition by gramicidin D. If one considers a maximum intravesicular volume of 4.5 $\mu$L/mg protein, this accumulation represented an internal proline concentration of 2.0–7.0 $\mu$M and a 10–20-fold concentration of proline in the incubation mixture. These values represent a minimum degree of concentration, since it is presumed that not all the vesicles in the population are actively accumulating proline.

Proline transport in membrane vesicles has several features that are similar to synaptosomal proline uptake. Among these features are the sodium ion dependence (Peterson & Raghupathy, 1972), substrate affinity and sodium activation (Peterson & Raghupathy, 1977), and sensitivity to the neurotoxins veratridine, *T. serrulatus* venom, and tetrodotoxin (Rhoads et al., 1982a,b). The sole source of energy to drive concentrative uptake under the experimental conditions established here was the artificially imposed transmembrane ion gradients. While other ions, notably $Cl^-$ and $K^+$, apparently contribute to proline uptake, the primary source of energy for uptake was the transmembrane sodium gradient ([$Na^+$] extravesicular > [$Na^+$] intravesicular). With intact synaptosomes, the ion gradients are presumably created by ($Na^+$,$K^+$)-ATPase, which accounts for the sensitivity of proline uptake to both ouabain and mitochondrial inhibitors (Rhoads et al., 1982a). It is likely that inhibition by CCCP and sensitivity of uptake to the absence of internal potassium are related to effects on the membrane potential. Intact synaptosomes have been reported to have a potassium diffusion potential (Blaustein & Goldring, 1975; Deutsch et al., 1981).

It is of interest to compare the properties of vesicular transport of proline with those previously reported for $\gamma$-aminobutyric acid (GABA), glutamic acid, and glycine. Kanner (1978) and Kanner & Sharon (1978), using a slightly different vesicular preparation, have shown that GABA transport can be driven by a $Cl^-$ gradient (out > in) in addition to a $Na^+$ gradient (out > in), while glutamic acid transport could be driven by a $Na^+$ (out > in) or a $K^+$ gradient (in > out). We have found similar results for the energetics of GABA and glutamic acid transport in our preparation (unpublished observations). The vesicular uptake of glycine was shown to be strictly dependent on the presence of $Na^+$ and $Cl^-$ in the incubation medium and could be driven by either a $Na^+$ gradient (out > in) or a $Cl^-$ gradient (out > in) when the other essential ion was present (Mayor et al., 1981). As we have shown here, vesicular proline transport could be driven only by a $Na^+$ gradient (out > in); $Cl^-$ and $K^+$ gradients are ineffective. Active transport of proline in vesicles isolated from bacterial cells (Amanuma et al., 1977a,b) was also driven by the electrochemical potential of a cation gradient. In this case, however, the cations were protons, and the transport was consistent with uptake by a proton-symport mechanism. The results of the present study are consistent with proline uptake coupled to a electrochemical gradient of $Na^+$ by a mechanism involving $Na^+$ symport.

The use of the vesicular transport system allows us to distinguish between some of the various mechanisms that may be responsible for the inhibitory effects of free fatty acids (FFA) on the synaptosomal uptake of proline. The inhibition of vesicular proline uptake by FFA showed the same specificity toward unsaturated fatty acids as previously seen in synaptosomes (Rhoads et al., 1982c). The fact that inhibition occurred under the defined energetic states employed for vesicle transport eliminates several possible sites of action of unsaturated FFA. Fatty acids have previously been shown to inhibit ($Na^+$,$K^+$)-ATPase activity (Ahmed & Thomas, 1971) and to uncouple mitochondrial oxidative phosphorylation (Heaton & Nicholls, 1976). In the present study, the role of these components was superseded by the artificial establishment of ion gradients, yet comparable inhibition of proline uptake by unsaturated FFA was observed in the absence of a requirement for these components. Hence, it is clear that they cannot be the primary sites of the inhibitory effects on proline uptake. Thus, unsaturated FFA may act directly on the proline carrier, or on the lipid domain surrounding the carrier, to affect its function or its coupling to the sodium gradient. Unsaturated free fatty acids have been shown to selectively associate with fluid lipid domains in membranes and to result in the differential disordering of these domains (Klausner et al., 1980). We suggest that the proline carrier is localized in a relatively fluid lipid domain and that agents such as unsaturated fatty acids that influence lipid–lipid or lipid–protein interactions in such a domain alter the function of the carrier. Conversely, the stimulatory effect of BSA on proline transport may be explained by a renormalization of the lipid domain by the removal of unsaturated free fatty acids. We have observed that the endogenous free fatty acid content of synaptosomal particles is considerably reduced following incubation with BSA (D. E. Rhoads, R. K. Ockner, N. A. Peterson, and E. Raghupathy, unpublished data).

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# $\beta_2$-Tubulin, a Form of Chordate Brain Tubulin with Lesser Reactivity toward an Assembly-Inhibiting Sulfhydryl-Directed Cross-Linking Reagent†

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ABSTRACT: $\beta_1$ and $\beta_2$ are the designations given to two forms of $\beta$-tubulin that have different electrophoretic mobilities on discontinuous polyacrylamide gels in the presence of sodium dodecyl sulfate [Little, M. (1979) *FEBS Lett. 108*, 283–286]. $\beta_1$ and $\beta_2$ constitute respectively 75% and 25% of the total $\beta$-tubulin in bovine brain. Although $\beta_1$ appears to be ubiquitous in animals, $\beta_2$ has so far only been found in the brains of cows, pigs, deer, rats, chicks, and dogfish but not in squid brain. $\beta_2$ is not found in bovine kidneys, in porcine lungs, or in any nonchordate tubulin that has been examined. When tubulin is reacted with the sulfhydryl-directed reagent *N*,*N*′-ethylenebis(iodoacetamide) (EBI), $\beta_1$, but not $\beta_2$, is converted to a faster moving form, $\beta^*$. The yield of $\beta_2$ in this reaction is not altered by the presence of drugs. When [$^{14}$C]EBI is used as a probe, most of the label is incorporated into $\beta_1$ rather than $\beta_2$. Tubulin molecules that have reacted with EBI to form $\beta^*$ are much less likely to polymerize into microtubules than are molecules that have not formed $\beta^*$. In view of the observation that only $\beta_1$, and not $\beta_2$, can form $\beta^*$, it is possible that $\beta_1$ represents a form of tubulin whose assembly may be regulated by a mechanism involving sulfhydryls. In contrast, $\beta_2$ may represent a form of tubulin whose assembly is regulated by some other mechanism.

Microtubules are long cylindrical organelles that play critical roles in a variety of cellular processes, including mitosis, secretion, axonal transport, and ciliary and flagellar motion (Roberts & Hyams, 1979). They are composed of a structural protein, tubulin ($M_r$ 100 000), a heterodimer of two $M_r$ 50 000 polypeptide chains (Ludueña et al., 1977), designated $\alpha$ and $\beta$. The hypothesis that there may be multiple forms of $\alpha$- and $\beta$-tubulins, with different amino acid sequences, has long been an attractive way to account for the different functional roles of microtubules and for the probable complexity of regulating their assembly in vivo (Fulton & Simpson, 1979). Evidence has been presented, on the basis of peptide mapping, that sea urchin eggs, embryos, and sperm contain a total of six forms of $\alpha$ and six forms of $\beta$ (Stephens, 1978); similarly, the existence of at least three forms of $\alpha$ and two of $\beta$, each with unique electrophoretic mobilities and isoelectric points, has been postulated in mammalian brains (Marotta et al., 1978, 1979a,b). In some of these cases, however, incomplete post-translational modifications or preparative artifacts cannot be rigorously excluded as possible explanations for the apparent heterogeneity. Recently, George et al. (1981), using calf brain tubulin prepared in a variety of ways, have found 17 isoelectric variants of tubulin. They suggest that some of the heterogeneity could arise from partial deamidation of glutamine and asparagine residues, a relatively common phenomenon in proteins (Robinson & Rudd, 1974). The clearest, most direct evidence for heterogeneity comes from the work of Ponstingl and his colleagues (Ponstingl et al., 1981; Krauhs et al., 1981; Little et al., 1981), who showed, by amino acid sequencing of the complete $\alpha$ and $\beta$ chains, that there exist at least four forms of $\alpha$ and two of $\beta$ in porcine brain tubulin. In none of the cases of tubulin heterogeneity that have been observed, however, have any of the structural differences between the different isotubulins been able to account for any kind of

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