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Effects of Free Fatty Acids on Synaptosomal Amino Acid Uptake Systems

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Abstract: The Na^+-dependent synaptosomal uptakes of proline, aspartic acid, glutamic acid, and γ-aminobutyric acid were strongly inhibited by monounsaturated fatty acids. With oleic acid, half-maximal inhibition was observed at about 15 μ*M*. The Na^+-independent uptakes of leucine, phenylalanine, histidine, and valine were less sensitive to inhibition by the unsaturated fatty acids. In contrast, the uptakes of all of these amino acids were unaffected by saturated fatty acids. The inhibition of proline uptake (and that of the other Na^+-dependent amino acids) by oleic acid was overcome by the addition of serum albumin and the data presented further indicate that the previously reported stimulation of proline uptake by albumin could be related to its fatty acid binding properties. **Key Words:** Synaptosomes—Amino acid uptake—Fatty acids. **Rhoads D. E. et al.** Effects of free fatty acids on synaptosomal amino acid uptake systems. *J. Neurochem.* **38,** 1255–1260 (1982).

In a previous paper (Peterson et al., 1979) we showed that bovine serum albumin (BSA) markedly stimulated the Na^+-dependent uptake of proline by synaptosomal fractions from rat brain cortices, and that several other proteins had no such effect. We also studied the effects of peptide fragments isolated from BSA and showed (Raghupathy et al., 1978) that the stimulatory effect on proline uptake was restricted to peptides isolated from the C-terminal region of the BSA molecule. The active site was further identified as the amino acid sequence 377–504 of the albumin molecule. Because the same sequence has been previously implicated (Reed et al., 1975) in the binding of long-chain fatty acids to BSA, we suggested that the stimulatory effect of BSA was related to its capacity to bind free fatty acids. Goto and Mizushima (1978) showed that a fatty acid preparation isolated from *E. coli* membranes inhibited proline uptake by *E. coli* membrane vesicles and that the addition of BSA overcame this inhibition.

More recently, we determined (Rhoads et al., 1982) that the stimulatory effect of BSA was not specific for proline but also occurred with other Na^+-dependent, veratridine-sensitive synaptosomal amino acid uptake systems [aspartic acid, glutamic acid, and γ-aminobutyric acid (GABA)]. On the other hand, the Na^+-independent uptakes of amino acids such as leucine, phenylalanine, and valine were unaffected. On the basis of this finding, we suggest that the site of action of BSA could be the Na^+-gradient that energizes Na^+-dependent transport of amino acids. In the present study we investigated the effects of representative saturated and monounsaturated fatty acids on several synaptosomal amino acid uptake systems. We show that the strictly Na^+-dependent amino acid uptake systems are highly sensitive to inhibition by monounsaturated fatty acids and that this inhibition is reversed by BSA.

EXPERIMENTAL PROCEDURES

Synaptosomal fractions were isolated from freshly excised cortices of adult Sprague-Dawley rats according to the procedure of Kurokawa et al. (1965). Protein contents of these fractions were measured by the method of Lowry et al. (1951). Unless otherwise stated, 0.1 mg of the synaptosomal protein fractions were incubated with ^{14}C

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Abbreviations used: BSA, Bovine serum albumin; GABA, γ-Aminobutyric acid; Medium TMNaK, Medium consisting of 10 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 150 m*M* NaCl, and 1 m*M* KCl.

amino acids (0.1 μCi) at 25°C in 1 ml of a medium consisting of 10 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 150 m*M* NaCl, and 1 m*M* KCl (medium TMNaK). Fatty acids were added at the required concentration in ethanol (10 μl). Control incubations received equivalent amounts of ethanol. Ethanol had no effect on the parameters studied. Other additions to the incubation media are described in the table and figure legends. Incubations were terminated by the addition of 5 ml of ice-cold buffer medium. The synaptosomal particles were harvested on Millipore filters (0.8 μm), washed with 20 ml of buffer at 4°C, and assayed for radioactivity as described elsewhere (Peterson and Raghupathy, 1972). When the filtered particles were washed with distilled water instead of the buffer medium, little or no radioactivity was retained on the filters, indicating that the amino acids were accumulated within membrane fractions susceptible to osmotic lysis. In some experiments crude synaptosomal-mitochondrial fractions [fraction P_2 of Gray and Whittaker (1962)] were used and the results were essentially the same as those obtained with the more purified synaptosomal fractions.

Materials

Uniformly labeled ^{14}C amino acids were purchased from New England Nuclear Corp. (Boston, MA) and had the following specific activities (mCi/mmol): proline, 260; aspartic acid, 219; glutamic acid, 254; GABA, 203; leucine, 298; phenylalanine, 536; histidine, 353; and valine, 225. Bovine serum albumin was obtained from Miles Labs., Inc. (Elkhart, IN). The fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Figure 1 shows that oleic acid (*cis*-9-octadecenoic acid) markedly inhibited the uptake of proline by rat brain synaptosomal fractions. Inhibition of proline uptake was evident at an oleic acid concentration as low as 5 μ*M* and was half-maximal at concentrations of about 15 μ*M*. The inhibition of synaptosomal proline uptake by oleic acid as a function of time is shown in Fig. 2. The extent of inhibition was essentially the same at all time intervals studied with no initial lag period. The effects of several saturated and unsaturated fatty acids on the synaptosomal uptakes of amino acids that are strictly dependent on external Na^+ (Peterson and Raghupathy, 1972; Blaustein and King, 1976) are shown in Table 1. At a concentration of 25 μ*M*, oleic acid inhibited the synaptosomal uptakes of aspartic acid,

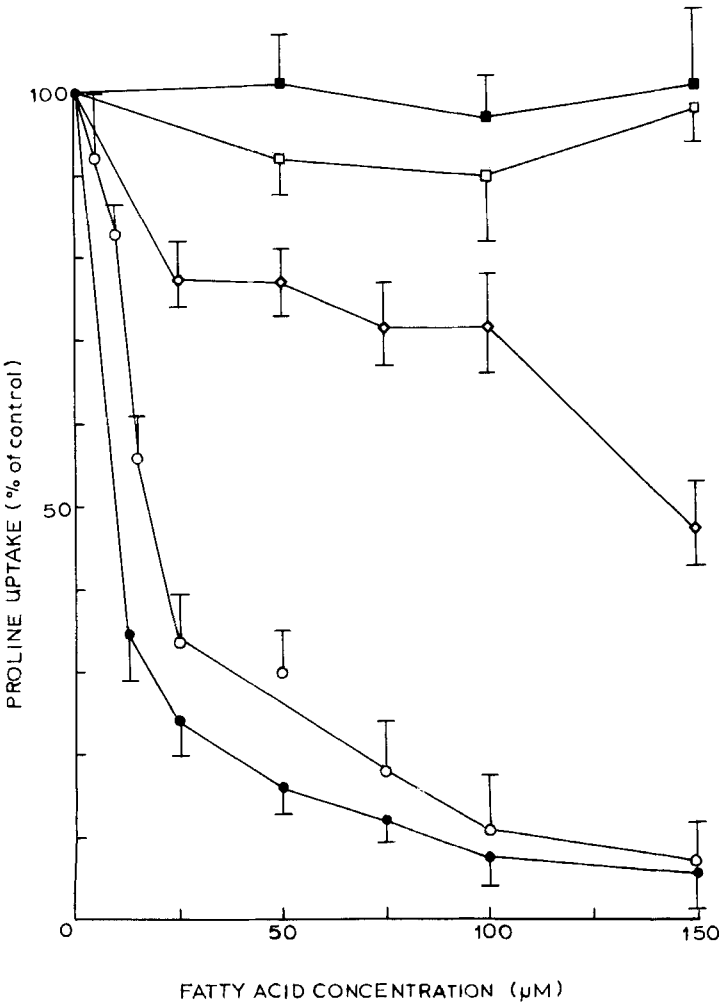

FIG. 1. Inhibition of synaptosomal proline uptake as a function of fatty acid concentration. Synaptosomal particles isolated from rat brain cortices were incubated with [^{14}C]proline in the presence and absence of 25 μ*M* fatty acid in ethanol. Incubation times were 10 min. The uptake of proline was measured as described in the text. Control uptake was 93.4 ± 11.8 pmol proline/mg protein/10 min. Values are expressed as % of control (uptake in the absence of fatty acid; about 1500 counts/min). Each value is the mean ± standard deviation derived from four to six experiments. (□), Palmitic acid; (■), stearic acid; (○), oleic acid; (●), palmitoleic acid; (◇), *cis*-11-eicosaenoic acid.

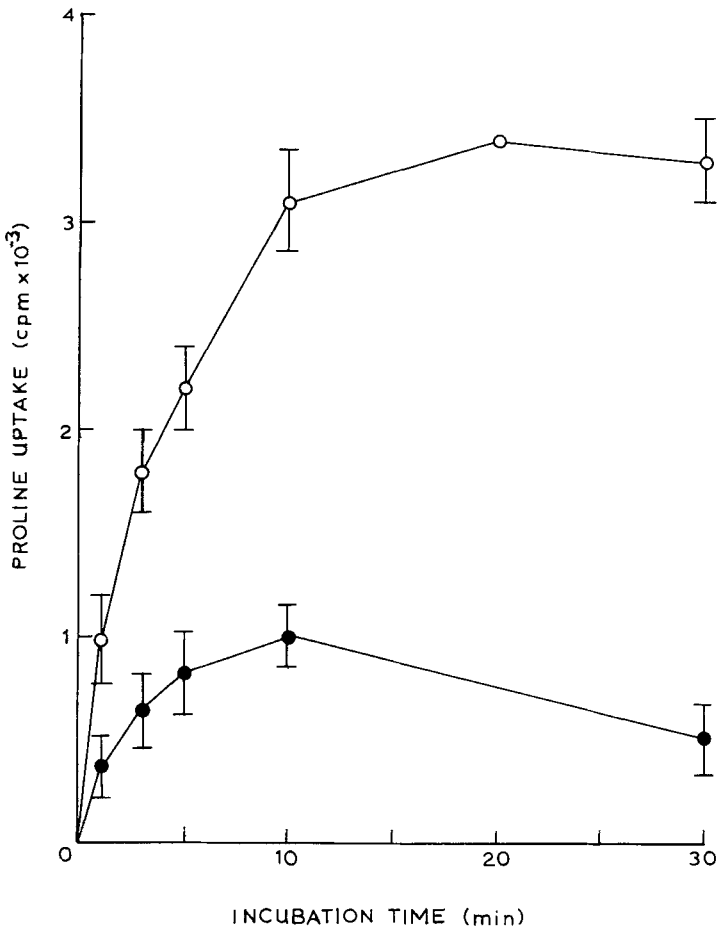

FIG. 2. Oleic acid inhibition of synaptosomal proline uptake as a function of time. Incubation conditions were as described in the legend to Fig. 1, except the incubation time was varied. Under these conditions, 1×10^3 cpm represents the uptake of 29.1 pmol proline/mg protein. Oleic acid was used at a concentration of 25 μM. Each point represents mean ± standard deviation derived from four to ten experiments. (○), Control; (●), oleic acid.

glutamic acid, and GABA as well as proline. Palmitoleic (*cis*-9-hexadecenoic) acid also strongly inhibited the uptake of proline, and, to a lesser extent that of aspartic acid, glutamic acid, and GABA. The dose-response curve for the inhibition of proline uptake by palmitoleic acid was similar to that of oleic acid, with half-maximal inhibition occurring at about 10 μM (Fig. 1). Another monounsaturated

TABLE 1. *Effect of free fatty acids on uptakes of amino acids by rat brain synaptosomal fractions*

Amino acid	Effect of fatty acids on amino acid uptake (% of control)				
	Oleic	Palmitoleic	Eicosaenoic	Palmitic	Stearic
Na^+-dependent					
Proline	34 ± 10 (6)	26 ± 4 (8)	78 ± 7 (4)	98 ± 13 (9)	101 ± 9 (5)
Aspartic acid[a]	19 ± 8 (6)	46 ± 5 (4)	82 ± 4 (6)	92 ± 7 (3)	102 ± 9 (4)
Glutamic acid[a]	12 ± 5 (6)	44 ± 6 (4)	68 ± 17 (6)	101 ± 14 (4)	104 ± 8 (4)
GABA	25 ± 3 (6)	66 ± 7 (4)	71 ± 9 (4)	103 ± 11 (4)	97 ± 6 (4)
Na^+-independent[b]					
Leucine	90 ± 8 (8)	92 ± 3 (4)	90 ± 6 (6)	101 ± 9 (9)	99 ± 8 (7)
Phenylalanine	96 ± 4 (8)	82 ± 12 (4)	92 ± 8 (3)	109 ± 5 (4)	102 ± 8 (4)
Histidine	99 ± 2 (4)	88 ± 8 (4)	103 ± 7 (3)	104 ± 7 (6)	106 ± 8 (4)
Valine	93 ± 3 (4)	95 ± 5 (4)	99 ± 7 (3)	104 ± 3 (4)	99 ± 3 (4)

Synaptosomal fractions were prepared from rat brain cortices as described in the text. Portions of the fractions were incubated at 25°C for 10 min in 1 ml of medium TMNaK with 0.1 μCi of ^{14}C-labeled amino acid in the presence and absence of 25 μM fatty acid. Values represent means ± standard deviations and the number of determinations is given in parentheses. Control values (picomol/mg protein/10 min) for the uptake under these conditions were: proline, 93 ± 12 (13); aspartic acid, 3567 ± 682 (10); glutamic acid, 1975 ± 487 (14); GABA, 1194 ± 418 (8); leucine, 67 ± 24 (22); phenylalanine, 83 ± 21 (14); valine, 82 ± 11 (8); and histidine, 85 ± 19 (8).

[a] Synaptosomal protein concentration was 0.01 mg/ml.

[b] Lack of inhibition by fatty acids was observed when the incubations were carried out in Na^+-free media as well.

fatty acid, *cis*-11-eicosaenoic acid, also inhibited the synaptosomal uptakes of proline, aspartic acid, glutamic acid, and GABA, but to a much lesser extent than did oleic and palmitoleic acids. In contrast with the monounsaturated fatty acids, two saturated fatty acids, palmitic (hexadecanoic) and stearic (octadecanoic), had no inhibitory effects on the uptakes of proline, aspartic acid, glutamic acid, and GABA.

The results presented in Table 1 also clearly show that none of the fatty acids, saturated or unsaturated, at concentrations of 25 μM had any effect on the synaptosomal uptakes of leucine, phenylalanine, histidine, and valine. We showed earlier (Peterson and Raghupathy, 1972) that the uptakes of these amino acids by synaptosomal fractions are not dependent on the presence of Na^+ in the incubation medium. Higher concentrations of the monounsaturated fatty acids produced slight inhibition; at concentrations of 75–100 μM oleic acid inhibited the uptake of leucine by about 25–30% (data not shown). The saturated fatty acids, however, had no effect at all on the concentrations investigated (0–150 μM). These results demonstrate that the Na^+-dependent amino acid uptake systems are more sensitive to inhibition by monounsaturated fatty acids than (1) by the saturated fatty acids and (2) are the Na^+-independent uptake systems.

The addition of 1 mg/ml (15 μM) BSA to the incubation mixture overcame the oleic acid inhibition of proline uptake up to an oleic acid concentration of 100 μM (Fig. 3). When BSA and oleic acid were added in an approximate molar ratio of 1:7, the uptake of proline was equivalent to that in control incubations (i.e., without oleic acid and BSA). At higher concentrations of oleic acid (> 100 μM), progressive inhibition of proline uptake was seen even in the presence of BSA (Fig. 3). In other experiments we had observed that the slight inhibition that occurred with leucine uptake at higher concentrations of oleic acid was also reversed by the addition of BSA. The addition of BSA reversed the inhibition of the uptakes of GABA, aspartic acid, and glutamic acid by oleic acid. Similarly, the inhibition of proline uptake by palmitoleic and eicosaenoic acids was overcome by the addition of BSA (data not shown).

DISCUSSION

The data presented in this paper clearly show that monounsaturated fatty acids strongly inhibit the Na^+-dependent uptakes of amino acids by rat brain synaptosomal fractions. The uptakes of Na^+-independent amino acids were less sensitive to inhibition by these fatty acids. On the other hand, saturated fatty acids had little or no effect on either the Na^+-dependent or -independent systems. The inhibition of proline uptake by oleic acid (and by other unsaturated fatty acids) was reversed by the addition of BSA; and, at a BSA:oleic acid molar ratio of 1:7, neither the stimulatory effect of BSA nor the inhibitory effect of oleic acid could be seen. In view of the results of Spector et al. (1969), who showed that BSA contains six primary sites for the binding of oleic acid, we can conclude that once these

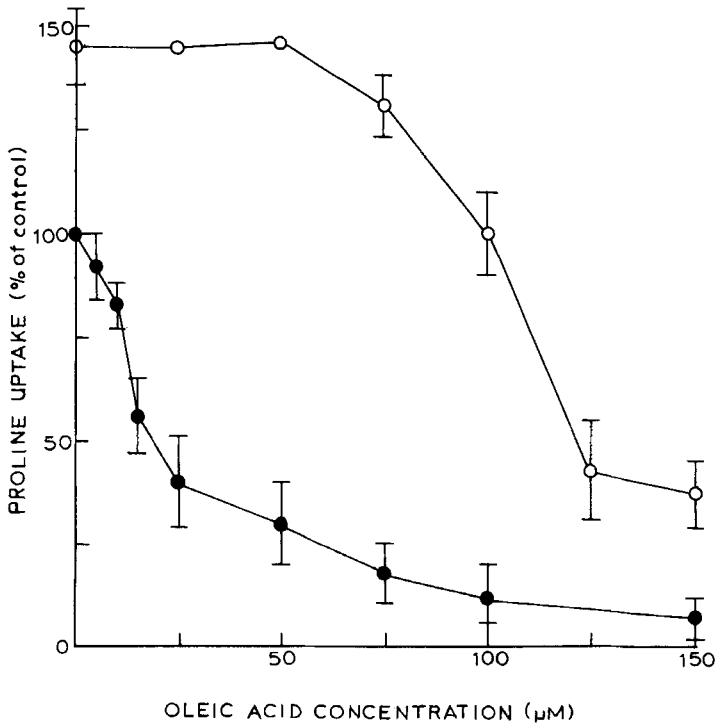

FIG. 3. Reversal of oleic acid inhibition of synaptosomal proline uptake by BSA. Incubation conditions were as described in the legend to Fig. 1. BSA was used at a concentration of 15 μM. Control uptake was 93.4 ± 11.8 pmol proline/mg protein/10 min. Each value is the mean ± standard deviation derived from four experiments. (○), BSA + oleic acid; (●), oleic acid.

binding sites are occupied the stimulatory effect of BSA on proline uptake is lost. These data strongly suggest that the reported stimulation by BSA of synaptosomal proline uptake (Peterson et al., 1979) is due to its complexing with inhibitory free fatty acids. These results extend the observations of Goto and Mizushima (1978), who have shown that a fatty acid preparation isolated from *E. coli* membranes strongly inhibited proline uptake into *E. coli* membrane vesicles and that BSA reversed this inhibition. In addition, these authors have shown a decreased concentration of free fatty acids in vesicle preparations incubated with BSA. From these data they concluded that free fatty acids inhibit the proline transport activity of *E. coli* vesicle preparations and that BSA reverses this effect by virtue of its fatty acid binding capacity.

The greater sensitivity of the Na^+-dependent amino acid transport systems to inhibition by unsaturated fatty acids can be interpreted in several ways. The site of fatty acid inhibition, or conversely, of BSA stimulation, may be through the action of unsaturated fatty acids on the Na^+ gradient, the origin of this gradient, the coupling of the gradient to the amino acid carrier, or the amino acid carrier itself. The latter possibility implies that free fatty acids affect some function of the amino acid carrier, such as the binding of substrates or the transfer of substrates across the membrane, which is fundamentally different between Na^+-dependent and Na^+-independent amino acid transport systems. The mechanisms of synaptosomal amino acid binding and transfer are not well understood. It is known that the physical state of the lipid domains has a profound effect on several membrane functions. Thus, free fatty acids may alter the physical state of the membrane and perturb vital lipid-protein interactions affecting carrier functions. Such an explanation may in addition account for the greater sensitivity to inhibition by unsaturated fatty acids by virtue of their greater solubility in the synaptosomal membrane.

With regard to the energetics of synaptosomal amino acid transport, it is known from studies with synaptosomal membrane vesicles that glutamic acid transport can be driven by artificially imposed Na^+ (out > in) or K^+ (in > out) gradients, and that this transport is stimulated by a membrane potential (interior negative) (Kanner and Sharon, 1978). Gradients of either Na^+ (out > in) or a number of small monovalent anions (out > in) have similarly been shown to drive the active transport of GABA in synaptosomal vesicle preparations (Kanner, 1978). Kinetic analyses have indicated that glutamic acid is cotransported with Na^+ (Bennett et al., 1973). Because of their amphipathic structure, free fatty acids may complex with cations and promote their transfer across lipid membranes. A proton ionophore function has been postulated for free fatty acids to account for their uncoupling of mitochondrial oxidative phosphorylation (Heaton and Nicholls, 1976). It is conceivable that free fatty acids inhibit Na^+-dependent transport by diminishing the Na^+ gradient via a direct Na^+ ionophoretic activity. Free fatty acids could also affect the synaptosomal Na^+ gradient by inhibiting the source of the gradient, the plasma membrane Na^+,K^+-ATPase. The Na^+,K^+-ATPase inhibitor ouabain inhibits a number of synaptosomal transport systems, including proline uptake (Rhoads et al., 1982). Ahmed and Thomas (1971) have reported 57% and 66% inhibition of rat brain microsomal Na^+,K^+-ATPase by 50 μM palmitoleic and oleic acids, respectively. In contrast, the saturated fatty acids (palmitic acid and stearic acid) were less than half as inhibitory at the same concentrations. The synaptosomal Na^+,K^+-ATPase could be inhibited directly in this fashion or indirectly by its dependence on ATP produced by the intrasynaptosomal mitochondria. As previously mentioned, free fatty acids are demonstrated uncouplers of mitochondrial oxidative phosphorylation (Heaton and Nicholls, 1976). Further, adenine nucleotide transport across the mitochondrial inner membrane has been shown to be inhibited by free fatty acids (Wojtczak and Zaluska, 1967). The net effect of such mitochondrial inhibition would be decreased intrasynaptosomal ATP and therefore decreased Na^+,K^+-ATPase activity. The present data do not allow us to distinguish between these possible sites of action. It is apparent that each of these potential sites is susceptible to inhibition by free fatty acids and that inhibition through any one mechanism might lead to the observed decrements in Na^+-dependent amino acid uptake.

It is well known that albumin has a protective effect on several membrane functions by virtue of its binding of inhibitory substances, presumably fatty acids. We show that one of these effects, the effect on synaptosomal amino acid transport systems, is linked with Na^+-dependent processes. In this event, endogenously occurring unsaturated fatty acids may modulate the Na^+-dependent transport of amino acids across the synaptic membrane. By the same reasoning, one might expect endogenous fatty acid-binding proteins, similar to those demonstrated in other tissues such as intestine and liver (Ockner and Manning, 1974; Mishkin et al., 1972), also to play a role in the regulation of the uptakes of these amino acids.

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REFERENCES

Ahmed K. and Thomas B. S. (1971) The effects of long chain fatty acids on sodium plus potassium ion-stimulated adenosine triphosphatase of rat brain. *J. Biol. Chem.* **246**, 103–109.

Bennett J. P. Jr., Logan W. J., and Snyder S. H. (1973) Amino acids as central nervous system transmitters: The influence of ions, amino acid analogues, and ontogeny on transport systems for L-glutamic and L-aspartic acids and glycine into central nervous synaptosomes of the rat. *J. Neurochem.* **21**, 1533–1550.

Blaustein M. P. and King A. C. (1976) Influence of membrane potential in the Na^+-dependent uptake of γ-aminobutyric acid by presynaptic terminals: Experimental observations and theoretical considerations. *J. Membr. Biol.* **30**, 153–173.

Goto K. and Mizushima S. (1978) Removal by bovine serum albumin of fatty acids from membrane vesicles and its effects on proline transport activity in *Escherichia coli*. *J. Biochem.* **84**, 251–258.

Gray E. G. and Whittaker V. P. (1962) The isolation of nerve endings from rat brain: An electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* **97**, 79–88.

Heaton G. M. and Nicholls D. G. (1976) Hamster brain-adipose tissue mitochondria. The role of fatty acids and the proton conductance of the inner membrane. *J. Biochem.* **67**, 511–517.

Kanner B. I. (1978) Active transport of γ-aminobutyric acid by membrane vesicles isolated from rat brain. *Biochemistry* **17**, 1207–1211.

Kanner B. I. and Sharon I. (1978) Solubilization and reconstitution of the L-glutamic acid transporter from rat brain. *FEBS Lett.* **94**, 245–248.

Kurokawa M., Sakamoto T., and Kato M. (1965) Distribution of sodium-plus-potassium stimulated adenosine triphosphatase activity in isolated nerve ending particles. *Biochem. J.* **97**, 833–844.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Mishkin S., Stein L., Gatmaitan Z., and Arias J. M. (1972) The binding of fatty acids to cytoplasmic proteins: Binding to Z proteins in liver and other tissues of the rat. *Biochem. Biophys. Res. Commun.* **47**, 997–1003.

Ockner R. K. and Manning J. A. (1974) Fatty acid binding protein in the small intestine. Identification, isolation and evidence for its role in cellular fatty acid transport. *J. Clin. Invest.* **54**, 326–338.

Peterson N. A. and Raghupathy E. (1972) Characteristics of amino acid accumulation by synaptosomal particles isolated from rat brain. *J. Neurochem.* **19**, 1423–1438.

Peterson N. A., Raghupathy E., Estey S. J., Tan C. H., and Salfi M. (1979) Stimulation of synaptosomal proline uptake by serum albumin. *J. Neurochem.* **33**, 739–743.

Raghupathy E., Peterson N. A., Estey S. J., Peters T., and Reed R. A. (1978) Serum albumin stimulation of synaptosomal proline uptake; partial identification of the active site. *Biochem. Biophys. Res. Commun.* **85**, 641–646.

Reed R. A., Feldhoff R. C., Clute O. L., and Peters T. (1975) Fragments of bovine serum albumin produced by limited proteolysis. Conformation of ligand binding. *Biochemistry* **14**, 4578–4583.

Rhoads D. E., Peterson N. A., Raghupathy E., and Verity M. A. (1982) Stimulation of Na^+-dependent, veratridine-sensitive synaptosomal amino acid uptakes by serum albumin. *J. Neurochem.* **38**, 1305–1310.

Spector A. A., John K., and Fletcher J. (1969) Binding of long-chain fatty acids to bovine serum albumin. *J. Lipid Res.* **10**, 56–67.

Wojtczak L. and Zaluska H. (1967) The inhibition of translocation of adenine nucleotides through mitochondrial membranes by oleate. *Biochem. Biophys. Res. Commun.* **28**, 76–81.