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Active Transport of Methadone in Synaptosomes

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SUMMARY

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The synaptosomal uptake of methadone was kinetically resolved into a diffusional and a saturable transport component. The latter process had an apparent K_m and V_{max} of 8.5 μM and 56.5 pmol/mg protein per 30 seconds, and led to synaptosomal accumulation of the drug. Lowering the temperature noncompetitively inhibited saturable transport which displayed a Q_{10} of 1.99. Incubation with 2,4-dinitrophenol had differential effects on methadone transport. While total uptake was slightly enhanced due to stimulation of the diffusional component, mediated transport was inhibited 93%. The pH dependence of uptake indicated that methadone was transported in its unionized form. The transport process was independent of sodium, lacked stereospecificity, and was insensitive to ouabain. In disrupted synaptosomes drug uptake was markedly diminished and the process lost its dependence on temperature and pH. Exodus of methadone from synaptosomes occurred rapidly, and reached equilibrium in 3 minutes. During efflux, countertransport of methadone was demonstrated. Methadone efflux was also transaccelerated by desipramine, levorphanol and pentazocine. Furthermore, these drugs, but not biogenic amines and morphine, competitively inhibited synaptosomal uptake of methadone displaying K_i values between 8 and 24 μM. The findings characterize an active transport system for methadone in synaptosomes, and provide evidence for sharing of the transport carrier by several other drugs, structurally related to basic amines.

INTRODUCTION

Only limited information is available on the transport of CNS acting drugs into synaptosomes. The synaptosomal uptake of amphetamines and 4-hydroxyamphetamine has been reported (1, 2). These compounds were apparently taken up by the catecholamine transport system, although some controversy seems to remain about the findings relative to amphetamine (2). The uptake of imipramine into synaptosomes has been attributed to binding, since the process was independent of time and temperature, and identical uptake was observed in disrupted synaptosomes (3). Similarly, the apparent uptake of dihydromorphine into synaptosomes was interpreted as a binding phenomenon (4). Synaptosomal uptake of mescaline has been described as an active process with a K_m and V_{max} of 1.23 μM and 26.34 pmol/mg protein per minute (5), not related to the catecholamine transport system. A preliminary report has been published on the localization of radiolabeled methadone in the synaptosomal frac-

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tion after incubation of the drug with brain homogenates, and after its injection *in vivo*. The content of radioactivity in the fraction diminished after rupturing the synaptosomes (6).

In the course of our work on the characterization of cellular transport mechanisms for drugs, we previously described processes for active transport of benzomorphans in leukocytes (7–10) and mediated uptake of chloroquine and other basic drugs in the isolated retina (11). The transport carrier in leukocytes also recognized methadone with a K_m of 20 μM. Our initial observations suggested that the transport of methadone in synaptosomes occurs in part by mediation (12). Subsequently, we presented preliminary evidence for the active nature of the mediated transport component (13). This paper describes the characterization of methadone transport in synaptosomes utilizing experimental criteria which allow a distinction between drug binding and transport, and resolve various forms of the latter process.

MATERIALS AND METHODS

Materials. d,*l* and *l*-1^{14}C-Methadone, ^{14}C-inulin, the tissue solubilizer Protosol and the scintillation mixture Omnifluor were obtained from New England Nuclear Corporation, Boston, Mass. 2-^{14}C-5,5-dimethyl-2,4-oxazolidine-dione,[2] was kindly provided by Dr. H. N. Christensen, Department of Biological Chemistry. The unlabeled drugs used in this study were gifts from Drs. H. H. Swain and H. J. Woods, Department of Pharmacology, The University of Michigan.

Isolation of synaptosomes. The scheme of the isolation procedure is shown in Fig. 1. Both the enriched synaptosomal preparation and a further purified synaptosome fraction were used in this study. The latter was obtained according to Autilio *et al.* (14), by density gradient centrifugation of the crude mitochondrial suspension at 25,000 × *g* for 60 min, as a layer at the interface of 7.5% and 10% Ficoll. However,

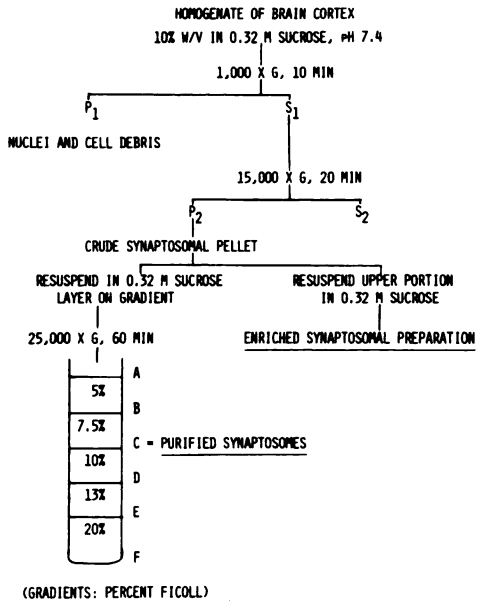


FIG. 1. *Outline of procedure for the isolation of synaptosomes*

predominantly the ESP fraction was utilized, and its detailed characterization including electron microscopy and the content of marker enzymes has been carried out and reported previously (15).

Viability of isolated synaptosomes. Synaptosomal content of potassium and sodium, and the uptake of oxygen were investigated after various times of incubation, reflecting experimental conditions encountered in this study. The ions were determined by flame photometry as described earlier (9). The rate of oxygen consumption was measured utilizing a YSI, model 53, oxygen electrode with air as the saturating gas. The procedure has previously been applied in our laboratory to measure the respiration of various cellular suspensions (16).

Synaptosomal volume and intracellular pH. Synaptosomal volume was estimated by subtracting inulin space from the total water space. The former was determined by incubating aliquots of the synaptosomal suspension containing approximately 1 mg protein/mg with 0.4 mg (0.1 μCi) of ^{14}C-inulin. After incubation for 5 min at 20°, the suspension was centrifuged for 4 min at 8,000 × *g* and the supernatant removed.

[2] The abbreviations used are: ^{14}C-DMO, 2-^{14}C-5,5-dimethyl-2,4-oxazolidine-dione; ESP, enriched synaptosomal preparation; DNP, 2,4-dinitrophenol.

The wet weight of pellet was determined to the nearest 0.01 mg, and the radioactivity in both the pellet and in an aliquot of the supernatant was measured by liquid scintillation spectrometry. Assuming that all of the ^{14}C-inulin in the pellet was extracellular, the amount of extracellular water was calculated and expressed on the basis of wet weight. Total water was determined by the difference between the wet and dry weights of synaptosomal pellets prepared by centrifugation as described above. The pellets were transferred to tared glass fiber discs and weighed to the nearest 0.01 mg. The samples were then dried overnight at 105° and reweighed. The difference in the two weights corresponded to the total water content. The difference between total and extracellular water was taken as the intracellular aqueous space. A correlation factor protein/wet weight allowed synaptosomal volume to be expressed in terms of protein content.

Intracellular pH was determined utilizing ^{14}C-DMO according to the method of Waddel and Butler (17). Aliquots of the synaptosomal fraction corresponding to approximately 1 mg protein were incubated with 10 μCi of ^{14}C-DMO in a total volume of 1.2 ml or 10 min at 20°. The mixture was centrifuged in conical polyethylene tubes (capacity 1.5 ml) for 4 min at 10,000 × *g*. The supernatant was decanted and its radioactivity determined by liquid scintillation spectrometry. After removing residual droplets of liquid in the centrifuge tubes by blotting with filter paper, the pellet was weighed, digested with Protosol and subjected to measurement of radioactivity. These results, together with the ratios wet weight/protein, total water/wet weight and the inulin space, were used to calculate the intracellular pH according to the formula described in the original publication of the method (17).

Determination of protein. An adaptation of the method of Lowry *et al.* (18) was utilized which was linear in the range 0.3 μg–5 μg (19).

Experiments on cellular transport. The basic procedure was similar to that described in our recent work on synaptosomal uptake of biogenic amines (15). Briefly, 50 μl aliquots of the synaptosomal suspension in 0.32 M sucrose, pH 7.4, were added to 850 μl of buffer medium and the suspension was incubated for 5 min at 20° under O_2. The uptake was initiated by the addition of 100 μl of buffer medium containing 0.05 μCi of radioactive methadone and sufficient unlabeled drug to give the desired final concentration. After 15–30 sec uptake was terminated by filtering the sample on previously washed Reeve-Angel, type 984H, glass fiber filter discs. The filters were further processed for liquid scintillation counting by digestion with Protosol as described (15). Mean efficiencies of the counting procedure for ^{3}H and ^{14}C, as determined by the method of external standardization, were 55% and 85%, respectively.

Drug efflux was investigated utilizing a quick dilution technique. Synaptosomal uptake was carried out by incubating 50 μl of the suspension with 100 μl of the ^{3}H-labeled drug, present at 5 μM, for 5 min at 20° under O_2. Drug exodus was initiated by rapidly diluting the incubation mixture with 850 μl of buffer medium. Exodus was terminated after 5–120 sec by rapid filtration of the samples. The filters were processed and subjected to liquid scintillation counting as outlined above and described previously (15).

The composition of the basic buffer medium used in the transport experiments was: TRIS, 35 mM; NaCl, 100 mM; KCl, 10 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 1 mM; sucrose, 25 mM; glucose, 10 mM. The pH of the medium was adjusted to 7.4 with HCl. The measured tonicity of the medium was 317 mOsm.

RESULTS

Characteristics of synaptosomal preparation. The preparation (ESP) predominantly used in this work (Fig. 1) was highly enriched in synaptosomes as determined by electron microscopy and biochemical characterization, involving the assay of acetylcholinesterase, lactic dehydrogenase and succinic dehydrogenase (15). Free mitochondria in the ESP represented less than 5% of all the vesicles present. Synaptosomal content of potassium and sodium in the preparation was 0.133 ± 0.03 μEq per mg

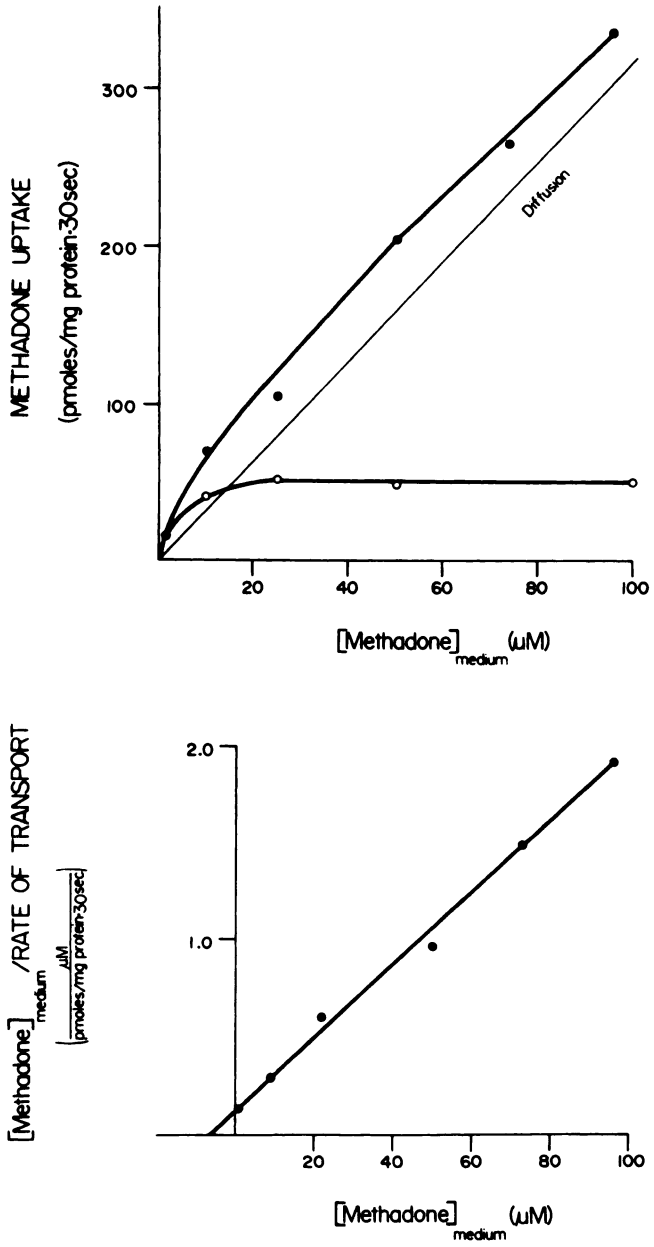


FIG. 2. *Resolution of synaptosomal methadone uptake*

Aliquots of the synaptosomal fraction were incubated in 900 μl of the standard buffer medium for 5 min at 20°. Uptake was initiated by the addition of 100 μl of buffer containing 0.05 μCi of ^{3}H-methadone and varying amounts of unlabeled drug. Incubations were terminated after 30 sec by rapid filtration of the suspension. The glass fiber discs (Reeve-Angel, 984 H) were quickly washed with 25 ml of ice-cold 0.9% NaCl (filtration time: 20–30 sec). In the upper graph plotted are the total uptake of methadone (●) and the total uptake minus diffusion (○). The diffusional constant of 3 pmol/mg protein per 30 sec corresponded to the slope of the linear portion of total methadone uptake, obtained at high concentrations (>100 μM) of the drug. The single reciprocal plot in the lower graph was obtained by utilizing data for the saturable uptake component. Each point is the mean of at least 12 determinations.

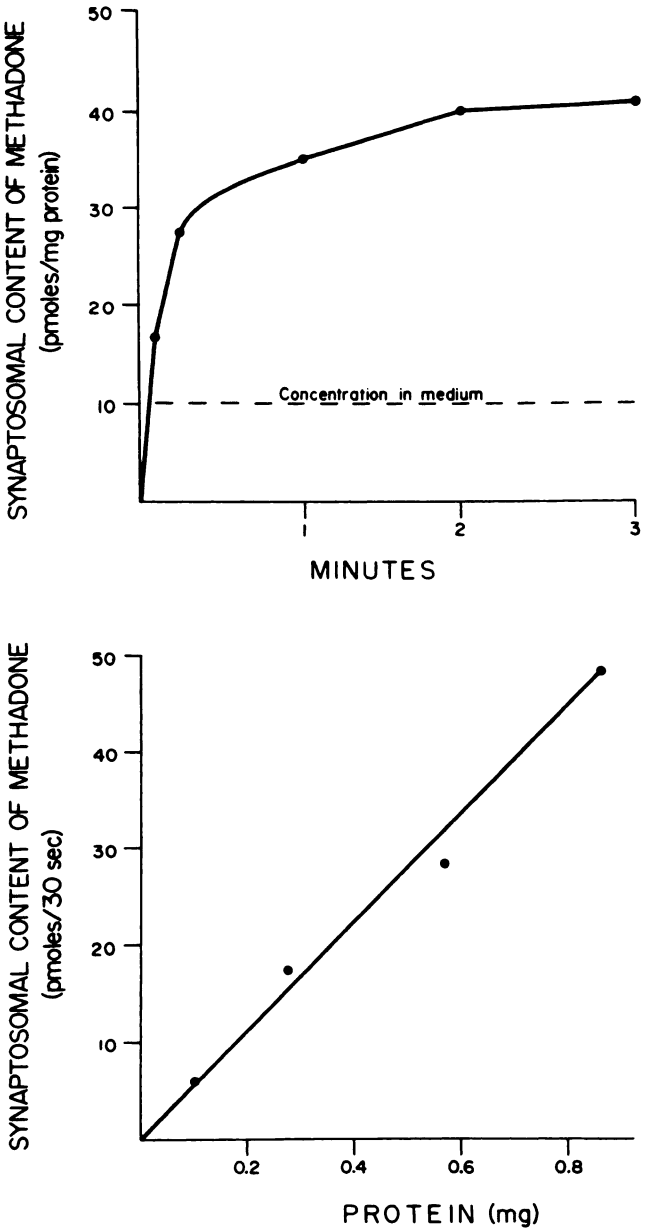


FIG. 3. *Dependence of methadone uptake on time and synaptosomal protein*

Aliquots of the synaptosomal fraction were incubated in 900 μl of the standard buffer medium for 5 min at 20°. The uptake was initiated by the addition of 100 μl of buffer containing 0.05 μCi of ^{3}H-methadone and sufficient amount of unlabeled drug to adjust the final concentration to 2 μM. Uptake was terminated at the times indicated (upper graph), or after 30 sec by rapid filtration of the suspension. The filters were washed and assayed for radioactivity as described. After digestion with Protosol for 1 hr at 60°, radioactivity was measured in a liquid scintillation spectrometer. Apparent uptake due to binding was determined in frozen and thawed samples, and has been subtracted from each point. Tissue concentration of methadone was calculated considering the relationship between vesicular volume and synaptosomal protein, determined as described under MATERIALS AND METHODS. Results of representative experiments are shown.

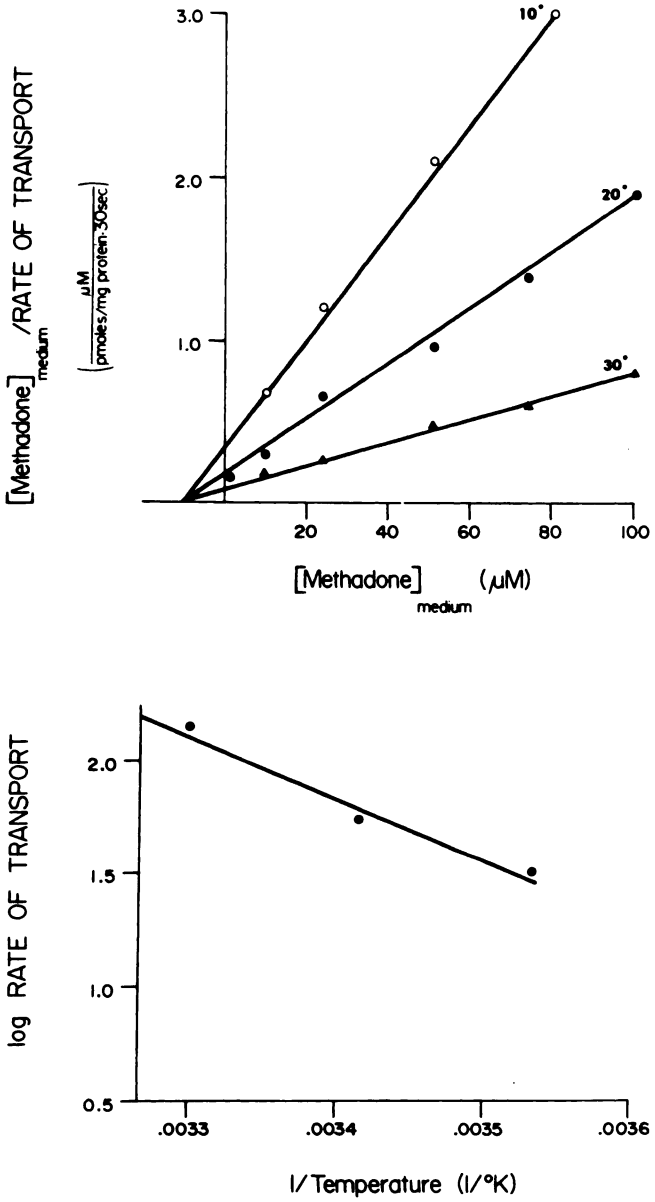


FIG. 4. *Effect of temperature on the uptake of methadone*

The initial rate of uptake of methadone was determined in aliquots of the synaptosomal fraction incubated for 5 min at the indicated temperatures (upper graph). The values for V_{max} were used to obtain the Arrhenius plot shown in the lower graph. The slope of the line in the latter plot corresponded to a Q_{10} of 1.99. Shown are mean values obtained in at least 3 experiments.

protein and 0.049 μEq per mg protein, respectively. Oxygen consumption was 20.74 ± 1.42 μl/mg protein per hr. These data were obtained after incubation of the ESP for up to 15 min at 20°. Synaptosomal pH and volume was 7.38 and 5.00 μl per mg protein. Yield of protein was 17.65 ± 1.49 mg per cerebral cortex.

Resolution of synaptosomal transport components for methadone. Total synaptosomal drug uptake was not saturable. At high concentrations of methadone synap-

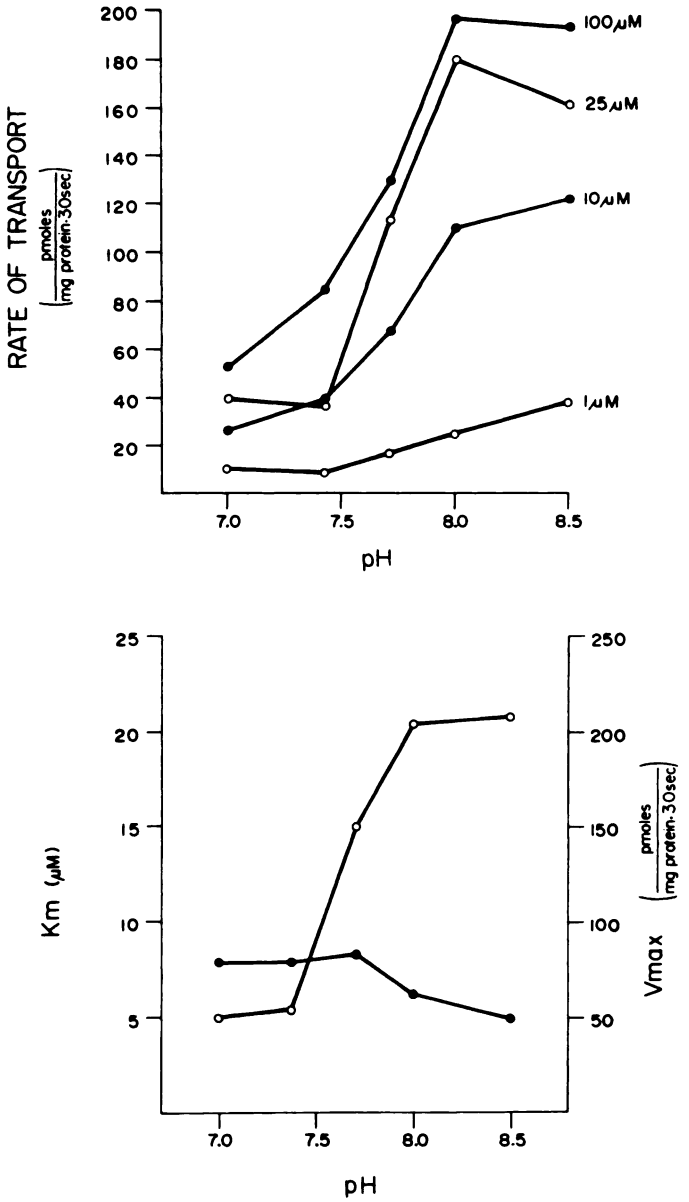


FIG. 5. *Effect of pH on the uptake of methadone*

Aliquots of the synaptosomal fraction were incubated for 5 min at 20° in the Tris-salts-sucrose medium adjusted to various pH. Subsequently, the initial rate of methadone uptake was determined at various concentrations of the drug as described (upper graph). In the lower graph, the values for K_m (●——●) and V_{max} (○——○), obtained from plots of (S)/V versus (S), are plotted as a function of the pH at which the uptake was determined. Each point is the mean of 4 determinations.

tosomal uptake was linear (Fig. 2) and exhibited a diffusional constant of 3 pmol/mg protein per 30 sec. When the contribution of the nonsaturable process was subtracted from total uptake, the saturable component of methadone transport was revealed. Apparent K_m and V_{max} values of the latter process were 8.5 μM and 56.5 pmol/mg protein per 30 sec (Fig. 2).

The dependence of the saturable uptake

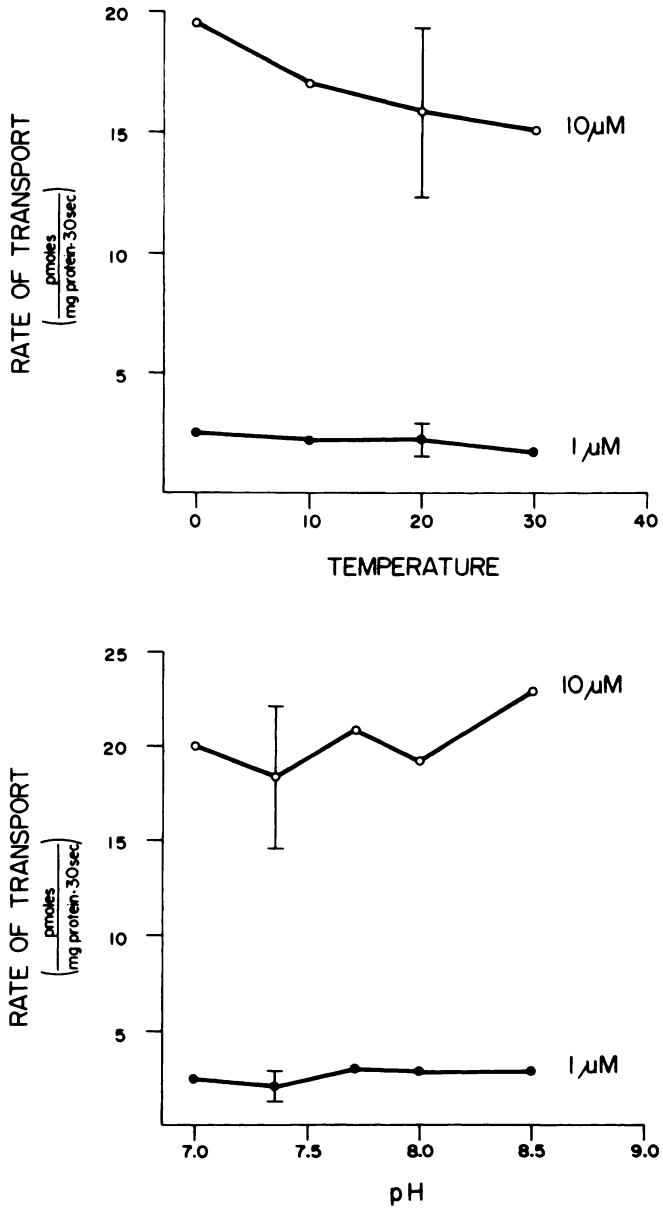


FIG. 6. *Temperature and pH dependence of the uptake of methadone in disrupted synaptosomes*

The synaptosomal preparation was subjected to repeated freezing at −70° and thawing. Subsequently, the initial rate of "uptake" of methadone (actually representing drug binding to the disrupted synaptosomes), present at concentrations of 1 μM or 10 μM was determined as described in legends to Figs. 4 and 5. Presented are the means and typical standard deviations of 4 determinations.

process on time and protein is shown in Fig. 3. Considering the above-outlined relationships, the following typical conditions for investigating drug transport were established: the uptake was terminated at 30 sec, approximately 0.5 mg protein of the synaptosomal fraction was used per observation, and the concentration of methadone was 2 μM, i.e., about 1/4 of its apparent K_m value.

Dependence on temperature. Lowering the temperature of incubation noncompet-

itively inhibited saturable methadone transport (Fig. 4). The Arrhenius plot of the process was linear, and its slope corresponded to a Q_{10} value of 1.99. At 0°, drug uptake was reduced to the level of nonspecific, i.e., not transport-related binding.

Dependence on pH. Rising pH accelerated the rate of methadone uptake. However, while V_{max} increased as a function of pH, the K_m was relatively insensitive to hydrogen ion concentration and did not change in the neutral pH region in which synaptosomal transport was typically investigated (Fig. 5).

Uptake in disrupted synaptosomes. Repeated freezing at −70° and thawing markedly altered the characteristics of synaptosomal methadone uptake. At concentrations of methadone approximating its K_m of uptake, drug-tissue association, which under these conditions represented drug binding, was reduced by 60% relative to control, and the process lost its sensitivity to temperature and pH (Fig. 6). In resolving the transport process for methadone, the binding component (freeze-thaw blank) was determined at each concentration of methadone and was subtracted from total drug uptake.

Effect of metabolic inhibitors. DNP enhanced total methadone uptake into synaptosomes. If, however, total uptake was resolved into a diffusional and saturable component as described above, the marked inhibition of the latter by DNP became apparent (Fig. 7). Concurrently, the diffusion coefficient after exposure to DNP increased up to 5-fold relative to control. Sat-

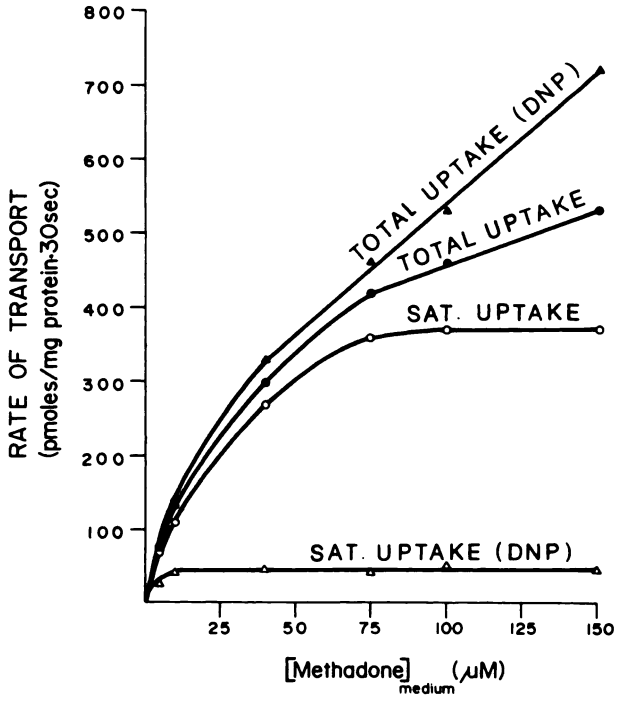


FIG. 7. *Effect of metabolic poisons on the uptake of methadone*

In these experiments the purified synaptosomal preparation described under MATERIALS AND METHODS was utilized. Aliquots of the fraction were incubated for 5 min at 20° in the standard buffer medium, or in buffer medium containing 2 mM DNP. Subsequently, the initial rate of uptake of ^{3}H-methadone was determined at various concentrations of the drug. Plotted are total uptake in absence (●) and presence (▲) of DNP, and the saturable uptake component in absence (○) and presence (△) of the inhibitor. The resolution of mediated transport from total drug uptake was carried out as described in the legend to Fig. 2. Values are means of 3–4 experiments.

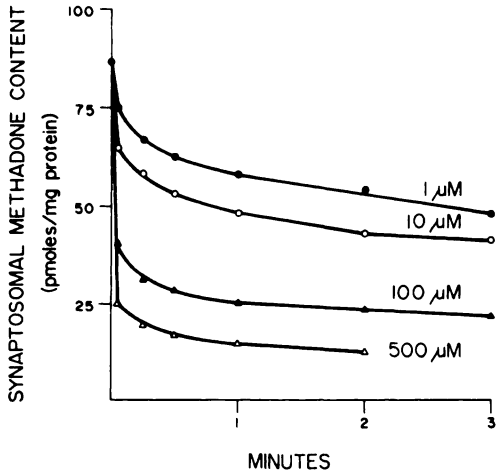


FIG. 8. *Transacceleration of methadone exodus*

Aliquots of the synaptosomal fraction were incubated with 8 μM ^{3}H-methadone for 5 min at 20°. Exodus was initiated by diluting the suspension 8-fold with buffer containing varying amounts of methadone. The final micromolar concentrations of methadone in the medium were 1, 10, 100, and 500. At the times indicated, exodus was terminated by rapid filtration of the suspension, and synaptosomal methadone was quantitated as described. As in most other experiments of this study, unaltered vesicular permeability was ascertained by determination of synaptosomal K^+ and Na^+. Shown are mean values of 4 experiments. The standard deviations around the means ranged between 6% and 14%.

urable methadone transport was also inhibited, although to a lesser degree (30%), by iodoacetamide.

Efflux of methadone and evidence for countertransport. Exit of radiolabeled methadone occurred rapidly and equilibrium was reached by 3 min following dilution of the uptake medium. The rate of methadone exodus was accelerated by increasing concentrations of unlabeled drug in the diluting medium (Fig. 8).

Transacceleration of methadone efflux by other drugs. The rate of methadone exodus was markedly transaccelerated by desipramine, levorphanol, and pentazocine present in the diluting medium (Fig. 9).

Inhibition of methadone uptake by CNS drugs. The CNS acting drugs which transaccelerated the efflux of methadone also competitively inhibited its synaptosomal uptake (Fig. 10). K_i values determined from Dixon plots (20) of the uptake data

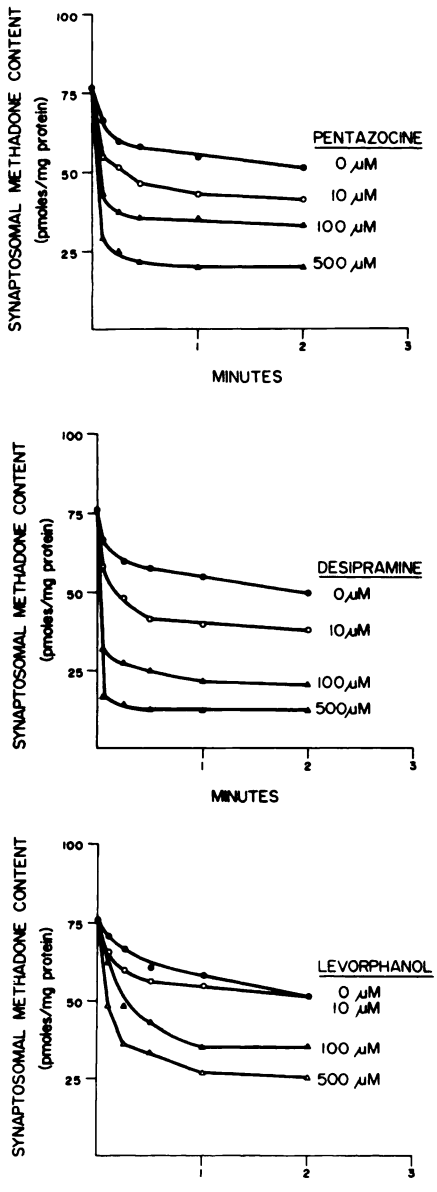
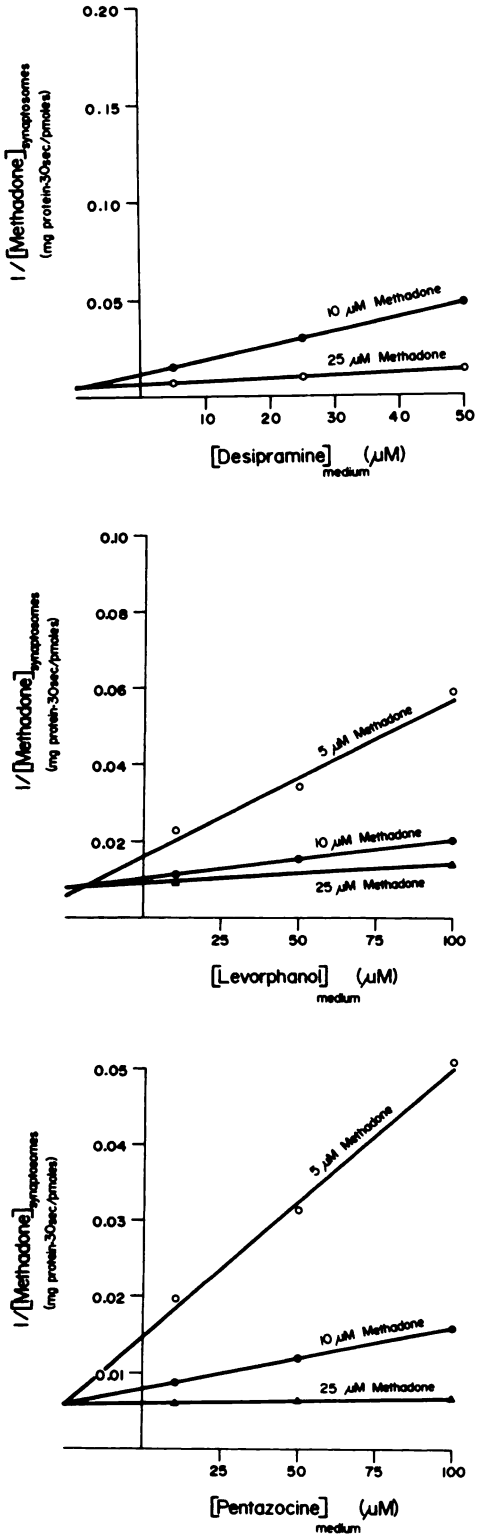


FIG. 9. *Transacceleration of the exodus of methadone by other CNS acting drugs*

Experimental conditions were as described in the legend to Fig. 8 except that the diluting medium contained varying amounts of desipramine, levorphanol, or pentazocine to give the final concentrations indicated. Presented are results of representative experiments.

were: desipramine, 12 μM; levorphanol, 23 μM; and pentazocine, 25 μM. Saturable uptake of methadone was not inhibited by 5-



hydroxytryptamine or norepinephrine even at concentrations severalfold higher than necessary to saturate the synaptosomal transport of these biogenic amines. Morphine also failed to inhibit the uptake of methadone.

Characteristics of methadone transport in purified synaptosomes. With one exception, these experiments yielded markedly similar results to those obtained with ESP. Methadone uptake reached saturation after 3–4 min and the K_m and V_{max} of the process were 12.78 μM and 471.94 pmol/mg protein per 30 sec. The latter value (Fig. 7) is considerably higher than that determined with the ESP. On the other hand, the similarity of findings includes the dependence on temperature and pH, the kinetics of exodus and its transacceleration by methadone, desipramine, levorphanol and pentazocine. The order of potency of the drugs in exhibiting the latter effect was that obtained with the ESP.

DISCUSSION

The utilized synaptosomal preparation had an enriched ratio of synaptosomes to mitochondria and favorable morphological and biochemical characteristics (15). The conditions of isolation and handling yielded a non-leaking synaptosomal membrane which exhibited unaltered permeability for potassium over a conveniently long time period. Nevertheless, several characteristics of methadone transport were also investigated in a synaptosomal preparation further purified by density gradient centrifugation. The data showed good agreement with findings obtained using the ESP which was predominantly applied in this work. A

FIG. 10. *Inhibition of the uptake of methadone by CNS acting for drugs*

Aliquots of the synaptosomal fraction were incubated for 5 min at 20° in the standard buffer containing various concentrations of desipramine, levorphanol, or pentazocine. The initial rate of radiolabeled methadone uptake was determined as described at three concentrations of the drug. Uptake due to diffusion was subtracted from total uptake, and the reciprocal of the initial rate of saturable transport was plotted against inhibitor concentration in the medium according to Dixon (20). Each point represents the mean of triplicate determinations in 3 separate experiments.

predictable exception was the considerably higher value for V_{max} in purified synaptosomes, apparently reflecting lower content of nonspecific protein in the preparation. This similarity of results affirms the feasibility of investigating synaptosomal transport phenomena in crude synaptosomal preparations. The latter have the advantage of superior viability over further purified synaptosomes (15) and have, therefore, been preferably chosen to study transport processes (e.g., 15, 21, 22). We would like to underline the importance of meticulously establishing and following experimental conditions which ascertain unaltered permeability of the plasma membrane in unicellular suspension utilized to study transport. Because of vesicular fragility, the aspect of permeability is of particular concern in investigating synaptosomal transport processes. We have previously described cellular potassium content and/or the ratio K^+/Na^+ as markedly sensitive indicators of a perturbed plasma membrane (23). The synaptosomes used in this work fulfilled this strict criterion of transport-related viability.

Methadone uptake into synaptosomes was the sum of both a saturable and a diffusional process. At methadone concentrations greater than about 25 μM, the nonsaturable component predominated. Under these conditions, a diffusional constant was calculated from the linear portion of the uptake curve. When the uptake due to the nonsaturable process was subtracted from total drug accumulation, the saturable process for the uptake of methadone became apparent. A similar resolution of the carrier-mediated transport component for chloroquine in the isolated retina was recently described (11). No pH gradient existed between the extra- and intracellular compartments, and the constancy of K_m in the pH region in which uptake was investigated suggested that methadone, with a pKa of 8.25, was transported in its unionized state. At equilibrium with 2 μM methadone in the external medium synaptosomal accumulation by the saturable transport was 5-fold. In addition to drug accumulation, the active nature of the mediated transport process was suggested by its temperature dependence, displaying a Q_{10} of 1.99.

In the course of attempting to fulfill an additional criterion for active transport, i.e., the inhibitory effect of metabolic poisons, DNP enhanced rather than inhibited synaptosomal methadone uptake. Such a stimulatory effect of DNP on the uptake of glucose and of α-aminoisobutyric acid into isolated tissue has previously been reported (24, 25). Furthermore, in the isolated rat retina, DNP markedly enhanced the uptake of chloroquine (11). The data of the latter study suggested that the effect of DNP was due to induced structural changes in the plasma membrane, resulting in increased permeability for chloroquine. The marked enhancement of the non-mediated uptake in retina prevented assessment of the effects of DNP on the saturable transport system. In synaptosomes, however, the resolution of the effect of DNP was successful. The enhancement of total methadone uptake by DNP was due to a facilitation of the diffusional component. Concurrently, saturable transport was inhibited 93% (Fig. 7). Apparently, and in agreement with our previous conclusions made in the study on retinal chloroquine transport, DNP induced membrane perturbation which resulted in enhancement of diffusional transport. In light of these findings, the earlier reported potentiating effects of DNP on transport in isolated tissue (24, 25) receive a molecular interpretation. It would be of interest to further investigate the mechanisms by which DNP elicits its effects on cell membranes. In the present study, the ascertained potent inhibition of mediated uptake by DNP, and to a lesser degree by iodoacetamide, represent important criteria in characterizing the process as active transport.

To distinguish between binding and transport of methadone, these phenomena were investigated in a synaptosomal preparation in which the vesicular structure has previously been abolished. The latter state was ascertained by lacking gradients for potassium and sodium. In such synaptosomal preparations, the uptake was low and

its characteristics were markedly altered. The residual radioactivity associated with the disrupted synaptosomal preparation was apparently due to binding of the drug. Nonstereospecific binding of methadone to albumin (26) and to tissue including synaptosomes has been described earlier (27). In these studies, percent binding of methadone to human plasma albumin was relatively independent of drug concentration, and the partition coefficients buffer/albumin and buffer/synaptosomes were virtually identical.

A strong criterion for ascertaining carrier-mediated transport as the molecular event under observation is the demonstration of countertransport in either direction of membrane translocation (28). Methodological reasons render the demonstration of countertransport during efflux easier than in the course of uptake (29). We have previously described this phenomenon during both uptake and exodus of CNS-acting drugs in leukocytes (9) and in the isolated retina (11). In synaptosomes, marked transacceleration of radiolabeled methadone exodus was obtained by unlabeled drug present in the external medium. Transacceleration of transmembrane transport of a given permeant by other molecular species suggests sharing of transport carriers by these compounds (29). While norepinephrine, 5-hydroxytryptamine, and morphine had no effect, desipramine, levorphanol and pentazocine facilitated methadone exodus from synaptosomes. Interaction with identical carrier binding sites of these compounds was further demonstrated by their competitive inhibition of methadone uptake. Biogenic amines had no effect on the latter process. It should here be emphasized, as we have done in discussing the kinetic relationship of the recently described drug transport system in the isolated retina (11), that future work should investigate and assess the effect on distribution kinetics of intracellular drug binding to macromolecular reactive sites.

In many of its properties the active transport of methadone in synaptosomes resembled the transport processes for basic CNS acting drugs described in leukocytes (9) and in the retina (11). On the other hand, the data presented strong evidence for dissimilarity of the system for methadone and that for biogenic amines in synaptosomes. The characteristics of the accumulative process for methadone described here underline the pharmacological importance of carrier-mediated drug transport. These systems can assume a regulatory role by altering the extracellular concentration and cellular content of drugs, thus affecting the equilibria of their interaction with membrane receptors and/or intracellular constituents (30).

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