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CATION-INDUCED ASYMMETRY OF CHOLINE FLUX ACROSS PRESYNAPTIC PLASMA MEMBRANES

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Summary

Highly cholinergic synaptosomes from the optic lobes of *Sepia officinalis* retain their ability to concentrate K^+ and extrude Na^+ and to synthesise acetylcholien in vitro. Choline uptake is hemicholinium-3 and Na^+ sensitive but is not obligatorily coupled to choline metabolism, or an energy supply as shown by the action of metabolic and ion pump inhibitors. The influx and efflux and/or steady-state distributions of choline in the presence of Na^+, Li^+, Rb^+, Cs^+ and mannitol were studied. The influx studies at different *cis*-choline concentrations revealed two systems for choline influx with different monovalent cation sensitivity and suggested a 1 : 1 interaction of choline with both mechanisms. Choline efflux was stimulated by *trans*-choline. Calculations of the internal/external concentration ratio expected if choline transport were coupled to the Na^+ gradient gave a maximal value of about 10^2. A secondary active transport of choline, where Na^+ is the driver solute provides an explanation for the cation sensitivity of the mechanism as well as for the method of coupling of choline transport to the varying demands of the nervous system for acetylcholine.

Introduction

The first study on the net uptake of choline into isolated presynaptic nerve endings from mammalian brain indicated that choline was transported across the limiting membrane of the presynaptic nerve terminal by a facilitative diffusion mechanism [1]. The results of more recent kinetic analyses of choline uptake into nerve endings from mammalian [2,3] and cephalopod tissue [4] are consistent with a kinetic model which involves two uptake mechanisms, usually referred to as the 'high' and 'low' affinity uptake systems. In nervous

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tissue the two putative uptake mechanisms appear to have somewhat different properties, for example; choline uptake at low (K_T 1 μM) choline concentrations shows greater substrate specificity [5] and Na^+ dependence [6]. There is also some evidence to suggest that low-affinity choline uptake is a property of all membranes [7—9]. Whereas high-affinity uptake may be specific to cholinergic membranes [10]. Although the necessity of an exogenous choline supply for the continued synthesis of acetylcholine in nervous tissue has been recognised for some time [11], the mechanism of coupling of the supply of choline to the varying demands of nervous tissue and the role of monovalent cations in this coupling is as yet unknown. The purpose of the present study was to investigate the role of the monovalent cations in the transport of choline with a view to formulating some working hypothesis for their involvement in the mechanism of choline transport across the synaptic plasma membrane. A nerve ending fraction known to be enriched in acetylcholine was prepared from the optic lobes of *Sepia officinalis* and partially purified for this purpose. Preliminary reports of some of this work have appeared [12,13].

Materials and Methods

Materials. Cuttlefish (*S. officinalis*) were supplied by the Institut de Biologie Marine, Arcachon, France, and kept in running sea water until required. Cellulose acetate membrane filters (type 11106, 25 mm diameter, 0.45 μm pore size) and a 30-fold filtration assembly were supplied by Sartorius Membran Filter GmbH, Göttingen, F.R.G., and Millipore Ltd. respectively. Thin-layer chromatography plates were supplied by Merck. Dry choline ((β-hydroxyethyl)-trimethylammonium hydroxide) and its acetate ester acetylcholine were obtained from Serva. $Na^{36}Cl$ solution (3 mCi/g Cl^-) and [*Me*-^{3}H]acetylcholine chloride (250 Ci/mol) were obtained from the Radiochemical Centre, Amersham, U.K. [*Me*-^{3}H]Choline chloride (4.2 Ci/nmol) was obtained from New England Nuclear. All isotopes were stored at −20°C and the purity of acetylcholine and choline was assessed by thin-layer chromatography. Choline esterase inhibitors, neostigmine ((3-dimethylcarbamoxyphenyl)trimethylammonium methyl sulphate), physostigmine (1′-methylpyrrolidine-(2′,3′,2,3)-1,3-dimethylindolin-5-yl-*N*-methylcarbamate) and paraoxon (diethyl-*p*-nitrophenyl phosphate) were supplied by Sigma Chemicals Co. Hemicholinium-3 (2,2′-(4,4′-biphenylen)-bis(4,4-dimethyl-2-hydroxymorpholinium bromide)) was supplied by Aldridge Ltd. Furosemide (4-chloro-*N*-(2-furylmethylsulphamyl)-*O*-anthranilic acid) was from Hoechst Pharmaceuticals.

Preparation of subcellular fractions. Presynaptic nerve terminals were prepared by a slight modification of the method used for obtaining synatosomes from the head ganglia of squid [14]. Sepiae were anaesthetised with 1% (v/v) ethanol in sea water and killed by decapitation. The optic lobes were removed, weighed and then homogenised in 0.7 M sucrose (1 g tissue/ml) with five up and down strokes of the pestle (clearance 0.25 mm) rotated at 840 rev./min in a Braun homogeniser unit. The homogenate was then centrifuged at 1000 × *g* for 11 min, the pellet retained (P_1) and the resulting supernatant fluid decanted and recentrifuged at 17 000 × *g* for 1 h in a Sorvall centrifuge (SS 34 rotor) giving three distinct fractions: a floating pellicle fraction (P_2L), an

opaque suspension (S_2) and a pellet (P_2H). Tissue suspensions were kept at 4°C throughout the fractionation procedure.

Enzyme and protein determinations. Enzyme activities were measured at 22°C in a Beckman Model 25 spectrophotometer. Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was determined by the method of Ellman et al. [15], fumarase (L-malate hydrolyase, EC 4.2.1.2) by the method of Racker [16], lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) as described by Whittaker and Barker [17], and choline acetyltransferase (EC 2.3.1.6) by the method of Fonnum [18]. Protein was determined after deposition on membrane filters as described elsewhere [19]. Briefly, this method involves the deposition of protein on cellulose acetate membrane filters followed by staining with amido black and then elution of the protein-dye complex.

Electron microscopy. The morphology of the isolated subcellular fractions was assessed either by obtaining a complete cross-section of the fixed pellet or by the filtration method of Baudhuin and Berthet [20]. Briefly, fractions were deposited on membrane filters, washed in incubation medium, fixed with glutaraldehyde, post-fixed with 1% OsO_4, dehydrated in a graded series of ethanol solutions and embedded in Epon via propylene oxide. Thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM 100B electron microscope.

Respiration and osmolarity. The oxygen uptake of the synaptosomal fraction was determined using an oxygen electrode (Rank Bros. Ltd., Cambridge). Measurements were carried out at 22°C and the results were computed as $\mu l \cdot min^{-1} \cdot mg^{-1}$ protein given that the incubation medium contained 0.224 μmol oxygen. Osmolarity was determined by depression of the freezing point using a Knauer osmometer.

Intrasynaptosomal ion contents. The floating pellicle fraction (P_2L) consisting largely of intact synaptosomes was decanted, washed by resuspension in 3 vols. of incubation medium and the synaptosomes sedimented at 43 000 × *g* for 10 ml (P_2L' fraction). The resulting pellet was rinsed once with 3 ml of the incubation medium which consisted of 423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 10 mM glucose, 10 mM Tris-HCl (pH 7.4), and then resuspended in the incubation medium (0.1 mg protein/ml). The synaptosomal suspension was then allowed to stand at 4°C for at least 15 min to recover from imposed osmotic stress [21] and then incubated for the desired periods of time, at 22°C. In studies involving inhibitors, synaptosomal suspensions were added to amounts of the dry test substances which gave the required final concentrations. At the specified times of incubation, aliquots were removed from the suspensions and transferred to 5 ml 1 M mannitol at room temperature, mixed and then poured onto moistened cellulose acetate membrane filters mounted on the manifold assembly. The filters containing the synaptosomes were then washed with 20 ml 1 M mannitol and the Na^+, K^+, $^{36}Cl^-$ and [^{3}H]choline contents of the synaptosomes determined from analyses of the trichloroacetic acid extracts of the filters [22,23]. Na^+ and K^+ in the extracts and the incubation media were determined using an Eppendorf flame photometer. Intrasynaptosomal $^{36}Cl^-$ and [^{3}H]choline were determined in separate experiments by scintillation counting of 1 ml aliquots in 10 ml of a

scintillation fluid consisting of 5 g PPO, 0.2 g dimethyl POPOP and 0.5 l Triton X-100 per l toluene. Counting efficiencies were determined by the channels ratio method. All counts were at least 10 times that of the background.

Acetylcholine determination. Total acetylcholine in the gradient fractions was extracted and stabilised by adjusting their pH to 4 with 0.1 M HCl and boiling for 10 min. For the determination of total intrasynaptosomal acetylcholine, synaptosomes were deposited on membrane filters and the filters were boiled for 10 min in 50 mM Tris-HCl, pH 4. The extracts were assayed on a small strip of the dorsal muscle of the leech, *Hirudo medicinalis*, as described elsewhere [17].

[^{3}H]Choline conversion. Intrasynaptosomal choline and acetylcholine were extracted from the synaptosomes on the filters with 2 ml of 0.02 M HCl and a 1 ml aliquot was taken for the determination of total ^{3}H radioactivity and another was dried by dessication after the addition of suitable amounts of carrier acetylcholine and choline. The dry acetylcholine and choline in the extracts were then solubilised with methanol, spotted on DC-cellulose thin-layer chromatography plates and separated by the *n*-butanol/ethanol/acetic acid/water (8 : 2 : 1 : 3, v/v) system and the spots visualised with Dragendorff's reagent. Spots were then scraped into scintillation vials and the acetylcholine and choline extracted and decolourised with 1 ml of 0.2% $Na_2S_2O_3$. The radioactivity as [^{3}H]choline and acetyl[^{3}H]choline was counted as described above. The recoveries of acetylcholine and choline were corrected for losses throughout the extraction procedure by the inclusion of radioactively labelled standards.

Choline flux. The P_2L synaptosomal fraction was washed by resuspension in 1 M mannitol, 0.1 mM paraoxon, followed by sedimentation at 41 000 $\times g$ for 10 min in a Sorvall (SS 34 rotor). The resulting pellet was then resuspended in a volume of 1 M mannitol, 0.1 mM paraoxon equivalent to the initial wet weight of optic lobe used (1 g/ml). For the measurement of choline influx, small aliquots of the synaptosomal suspension were transferred into the incubation medium, which, in the control condition consisted of 423 mM NaCl, 9 mM KCl, 9.22 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.5 mM $MgSO_4$, 10 mM glucose, 10 mM Tris-HCl (pH 7.4), and 0.1 mM paraoxon. In experiments with Cs^+, Rb^+, K^+ and Li^+ these ions replaced Na^+ on an equimolar basis. Unless otherwise stated tissue suspensions (0.1 mg protein/ml) were preincubated for 5 min before the addition of choline which gave the desired final concentration. At the specified times of incubation, synaptosomes were isolated from the incubation media by rapid filtration on cellulose acetate membrane filters [22,23]. This procedure involved the transfer of the synaptosomes into 10 ml of 1 M mannitol followed by their deposition on membrane filters. Initial rates of uptake were measured by taking aliquots of the suspension after 10 min of incubation at which time uptake was linear. The [^{3}H]choline in the synaptosomes was determined by extraction of the filters with 2 ml of trichloroacetic acid followed by scintillation counting. For the measurement of choline efflux, synaptosomes were incubated for at least 60 min in the control medium with the addition of [^{3}H]choline (0.48 μM, 2 μCi/ml) at zero time. At 60 min of incubation 1 ml aliquots of the suspensions were removed, deposited on membrane filters as described above and the filters were transferred to 2 ml of the appropriate incubation medium in 15-ml glass vials. Efflux was measured

by the removal of small aliquots of medium at the specified times of incubation. No protein was detectable in the incubation medium under the conditions of these experiments.

Statistical analyses. Different samples were compared by Student's *t*-test (two-tailed) where $P < 0.05$ was taken as the level of significance.

Results

Assessment of gradient fractions

Subcellular fractions were assessed by marker studies and electron microscopy. The results of the enzyme marker studies are shown in Table I. The P_2L fraction was enriched in acetylcholine, cholinesterase, choline acetyltransferase and occluded lactate dehydrogenase, whereas the P_2H fraction showed a substantial enrichment in the mitochondrial marker fumerase. These results are in agreement with those reported for the subcellular fractionation of squid head ganglia [17]. Electron microscopical examination of the P_2L fraction (Fig. 1a) showed that this fraction was highly enriched in nerve ending particles (synaptosomes). Some of the synaptosomes contained dense-cored vesicles and several occluded mitochondria (Fig. 1b). There are few free mitochondria in the P_2L fraction whereas the P_2H fraction consists mainly of free mitochondria (Fig. 1c and d). A mean synaptosomal diameter of 1.16 μm was calculated from the measurement of the major and minor axes of 300 synaptosomal profiles and is similar to that of squid synaptosomes [17] and octopus synaptosomes [24] but somewhat larger than the mean diameter of synaptosomes isolated from the mammalian cerebral cortex [25].

TABLE I

DISTRIBUTION OF MARKERS IN SUBCELLULAR FRACTIONS FROM *SEPIA* OPTIC LOBES

The fractions were prepared and assayed for the markers as described in Materials and Methods. The results are averages of two separate experiments and are given as relative specific activities (RSA = percent recovered activity/percent recovered protein in the same fraction). Homogenate-specific activities were: protein, 92 mg/g original tissue; lactate dehydrogenase, 3.9 nmol/min per mg protein; acetylcholinesterase, 18.3 μmol/min per mg protein; choline acetyltransferase, 32 nmol/min per mg protein; fumarase, 0.032 *A* units/min per mg protein; acetylcholine, 6.3 nmol/mg protein.

Markers	Fractions								
	P_1		P_2L		S_2		P_2H		Recovery (%) **
	RSA	% *	RSA	% *	RSA	% *	RSA	% *	
Protein		32		12		36		20	96
Occluded lactate dehydrogenase	0.8	25.6	1.2	14.4	1.5	54	0.5	10	80
Acetylcholinesterase	0.5	16	1.8	21.6	0.9	32.4	1.4	28	92
Choline acetyltransferase	0.4	12.8	1.5	18	1.9	68.4	1.6	32	78
Fumarase	0.2	6.4	1.3	15.6	0.6	21.6	2.9	58	79
Acetylcholine	0.3	9.6	2.9	34.8	0.9	32.4	0.9	18	63

* Percent of total activity in all fractions analysed.

** Percent activity recovered in all fractions from total homogenates.

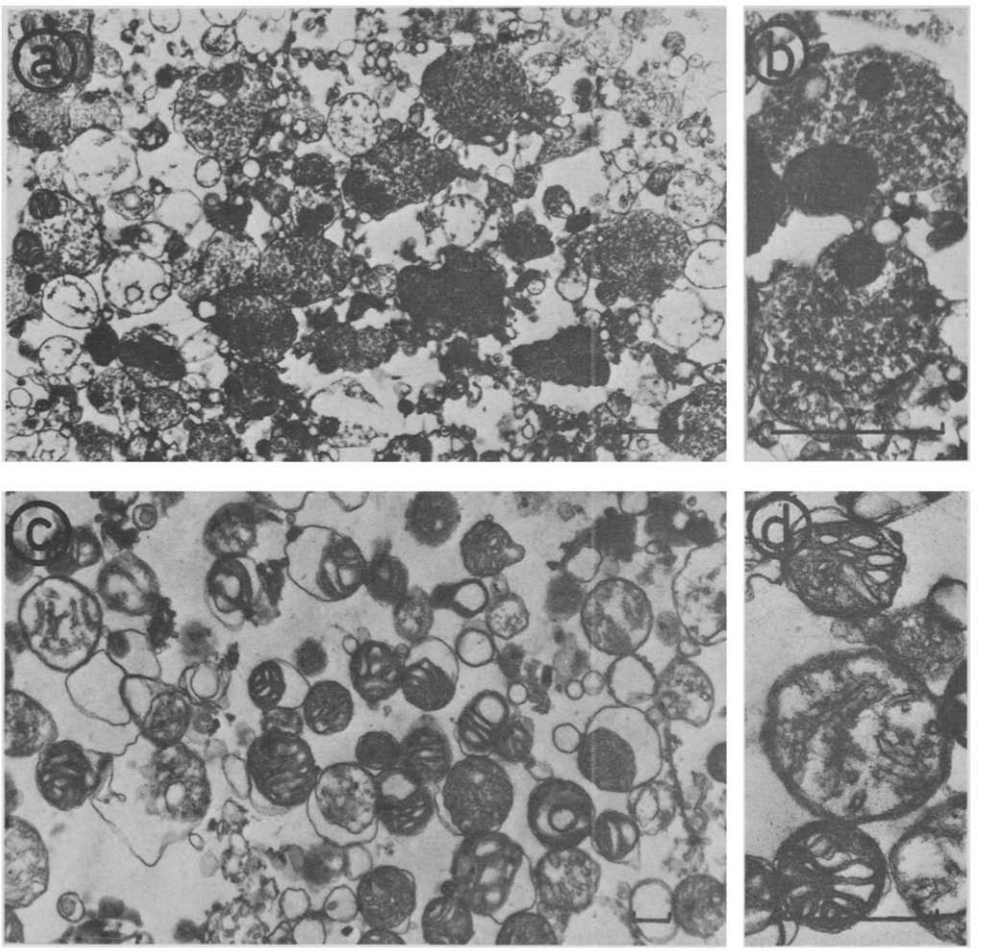

Fig. 1. Electron micrographs of the subcellular fractions. (a) The P_2L fraction after equilibration with the incubation medium. A predominance of nerve ending particles is found. (b) Occluded mitochondria and synaptic vesicles are easily recognisable at higher magnification. (c) The P_2H fraction containing predominantly mitochondria. (d) At higher magnification cristae are clearly visible. Bars indicate 1 μm (a and b) and 0.1 μm (c and d).

Effects of incubation time on the nerve ending fraction

Several parameters of the nerve ending fraction were analysed. A relatively slow gain of Na^+ and loss of K^+ was found as a function of the time of incubation (Table II). At 120 min of incubation the K^+ content of the synaptosomes was decreased by 40% relative to the zero-time point whereas the Na^+ level was about 40% greater after 120 min of incubation than at zero time. Relative to mammalian synaptosomes Sepia synaptosomes contained a large amount of acetylcholine (Table II). The level of the acetylcholine in the synaptosomes was depleted on dilution of the P_2L fraction 1 : 10 (v/v) with incubation medium followed by centrifugation to form a pellet which was then left at 4°C for 20 min (Table II). When exogenous choline (0.48 μM) was added to the acetylcholine-depleted synaptosomes, the acetylcholine content of the fraction increased as the time of incubation increased with a maximal rate of increase

TABLE II

EFFECTS OF LONG PERIODS OF INCUBATION ON PARAMTERS OF THE NERVE ENDING PARTICLES

Suspensions of nerve ending particles were incubated for varying periods of time in artificial sea water at 22°C with the addition of [^{3}H]choline (0.48 μM, 2 μCi/ml) at zero time where applicable. At the chosen times, aliquots in triplicate were removed, filtered and the Na^+, K^+ and acetylcholine contents and [^{3}H]-choline conversion in the nerve ending particles determined as outlined in Materials and Methods. Results are the mean ± S.D. of the number of separate experiments in parentheses or the average of two independent experiments for the acetylcholine studies. —, not determined. % choline conversion = 100 × dpm acetyl[^{3}H]choline/total dpm.

Time (min)	μmol/g protein		% choline conversion	Acetylcholine (μmol/g protein)	
	Na^+	K^+		Depleted	Control
0	244 ± 19 (7)	150 ± 17 (7)	0.2 ± 0.2 (4)	0.8	9.1
5	—	—	2.6 ± 3.1 (4)	0.8	9.1
10	267 ± 28 (7)	126 ± 20 (6)	3.8 ± 1.2 (4)	1.75	9.2
15	—	—	3.9	1.85	9.2
20	307 ± 25 (6)	104 ± 19 (6)	7.1 ± 1.5 (3)	2.4	9.3
25	—	—	11.7 ± 1.0 (4)	2.2	8.1
40	338 ± 37 (6)	105 ± 9 (6)	7.8 ± 2.0 (3)	5.4	7.1
50	409 ± 38 (3)	107 ± 10 (5)	5.7 ± 1.0 (3)	5.8	8.2
60	375 ± 25 (3)	94 ± 15 (4)	6.2 ± 1.0 (3)	5.8	8.4
80	381 ± 35 (3)	94 ± 17 (4)	—	—	—
100	333 ± 25 (4)	89 ± 18 (4)	5.5 ± 1.0 (3)	—	—
120	339 ± 20 (4)	89 ± 12 (4)	—	—	7.2

from 20 to 30 min of incubation. Under similar conditions (Table II) the control synaptosomes lost a small amount of acetylcholine over the 120 min incubation time. The results indicate that *Sepia* synaptosomes are capable of replenishing their stores of acetylcholine. Studies of the conversion of [^{3}H]-choline to acetyl[^{3}H]choline in the control condition showed a maximum conversion of [^{3}H]choline (0.48 μM) to acetyl[^{3}H]choline of 15% at 30 min of incubation. At shorter or longer incubation periods the percentage conversion was considerably smaller (Table II).

Effects of various incubation conditions on the intrasynaptosomal ion contents

The specificity of action of various pharmacological agents was studied by analyses of the Na^+, K^+, $^{36}Cl^-$ and [^{3}H]choline contents of the synaptosomes (Table III). Kinetic studies of [^{3}H]choline and $^{36}Cl^-$ interaction with the synaptosomes showed characteristic uptake curves in each condition studied (Fig. 2). Uptake was eliminated and Na^+ and K^+ in the synaptosomes decreased by lysis of the synaptosomes (Table III). Hemicholinium-3 (0.1 mM) decreased choline uptake by 67% whereas the Na^+, K^+ and chloride levels were not significantly different from the control. Ouabain (1 mM), CN^- + iodoacetate (2.5 mM) or *N*-ethylmaleimide (1 mM) led to a collapse of the Na^+ and K^+ gradients and at most a 38% inhibition of choline uptake. Dithiothreitol (1 mM) had negligibly small effects on the ion contents of the synaptosomes whereas the mercurials resulted in decreases of the Na^+, K^+, $^{36}Cl^-$ and [^{3}H]-choline contents of the synaptosomes, the action of the compounds being dose dependent. Choline esterase inhibitors (lipid-soluble paraoxon, physostigmine

TABLE III

EFFECTS OF INCUBATION CONDITIONS ON THE Na^+, K^+, Cl^- AND CHOLINE LEVELS IN THE SYNAPTOSOMES

Suspensions of synaptosomes were incubated in artificial sea water for 40 min at 22°C with the addition of the substances indicated. [^{3}H]Choline (2 μCi/ml, 0.48 μM) and $^{36}Cl^-$ (10 μCi/ml) were added at zero time. After this time triplicate aliquots of the suspensions were removed, the synaptosomes separated from the incubation media by rapid filtration and the amount of chloride, [^{3}H]choline, Na^+ and K^+ in the synaptosomes determined. Results are from three to six separate experiments where S.D. is less than 10% and are expressed as a percentage of the control in the same experiment. All values are corrected for the small amount of binding of ions to the filters found by filtration of the media alone. In these experiments the mean values for the control were Na^+, 262; K^+ 94; Cl^-, 421, 1.4 μmol/g protein. —, not determined.

Condition	Concn. (mM)	Na^+ (%)	K^+ (%)	Cl^- (%)	Choline (%)
Hemicholinium-3	0.1	91	119	70	33
Ouabain	1	151	52	113	73
CN^- + iodoacetate	2.5	141	57	97	77
Furosemide	1	72	50	83	55
N-Ethylmaleimide	1	150	44	112	62
Iodoacetate	1	52	39	54	58
$HgCl_2$	1	11	15	11	1.2
$HgCl_2$	0.1	—	—	10	1.4
$HgCl_2$	0.01	—	—	55	61
$HgCl_2$	0.001	—	—	84	88
Methylmercuric	1	25	0	13	2
Phenylmercuric	1	49	37	47	2
p-Mercuribenzoate	1	25	14	18	1.1
Dithiothreitol	1	102	94	102	93
Neostigmine	0.1	93	121	—	98
Paraoxon	0.1	96	190	99	90
Physostigmine	0.1	94	98	84	104
CsCl replaced NaCl		—	—	92	—
Lysis		15	14	16	5

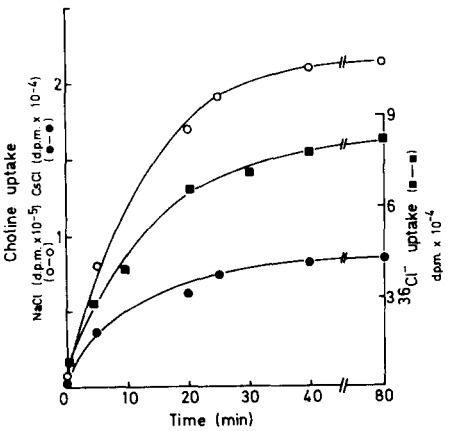

Fig. 2. The net uptake of $^{36}Cl^-$ and [^{3}H]choline. Synaptosomal suspensions were incubated in the chosen media at 22°C with the addition of either [^{3}H]choline (0.48 μM, 2 Ci/ml) or $^{36}Cl^-$ (10 μCi/ml) at zero time. After the chosen incubation periods triplicate samples were removed, the synaptosomes separated from the incubation media by rapid filtration and their chloride and choline content determined as $^{36}Cl^-$ and [^{3}H]choline, respectively. Results for the net uptake of [^{3}H]choline in the control condition (○———○) and when CsCl replaced NaCl (●———●) are the average of triplicate samples in a typical experiment and the data points for chloride uptake (■———■) are the means of four separate experiments where S.D. is less than 7%.

TABLE IV

MONOVALENT CATION DEPENDENCE OF [^{3}H]CHOLINE UPTAKE AT LOW AND HIGH *cis*-CHOLINE CONCENTRATIONS

Incubation and filtration conditions were as described in Fig. 2. Results (± S.D., $n = 3$) are expressed as a percentage of the control (NaCl medium) in the same experiment. Cations and mannitol replaced NaCl on an equimolar basis. —, not determined.

Condition	[^{3}H]Choline uptake (% control)	
	Media choline concentration (μM)	
	0.48	102
LiCl	34 ± 2	103 ± 2 *
KCl	3 ± 0.5	35 ± 4
RbCl	2 ± 0.3	32 ± 5
CsCl	1.6 ± 0.5	24 ± 7
500 mM mannitol	80 ± 3	—
750 mM mannitol	73 ± 1	—
1 M mannitol	65 ± 4	—

* Not significantly different from the control.

and poorly lipid-soluble neostigmine) which are often included in the incubation media to minimise the production of choline from exogenous acetylcholine had no apparent effects on the Na^+, K^+, $^{36}Cl^-$ or [^{3}H]choline contents of the synaptosomes.

Effects of monovalent cations on [^{3}H]choline uptake

The first indication that the transport of [^{3}H]choline into *Sepia* synaptosomes was affected by the replacement of Na^+ with other monovalent cations from an analysis of the uptake of [^{3}H]choline (Fig. 2, Table IV). When Na^+ was replaced by the other monovalent cations both the initial rate of uptake (Fig. 2) and the equilibrium level of [^{3}H]choline in the synaptosomes were reduced. This effect was found at both high (102 μM) and low (0.48 μM) *cis*-choline concentrations. Under similar conditions the compartmentation of $^{36}Cl^-$ was not affected (Table III). The effects of Na^+ substitution were more marked at low *cis*-choline concentration than at high concentration, although the order of inhibitory potency of the cations was similar at both choline concentrations, where $Cs^+ > Rb^+ > K^+ > Li^+ > Na^+$. When mannitol replaced NaCl a reduction of choline uptake was found, this being smaller than that found when the monovalent cations were substituted for Na^+ (Table IV).

Cation dependence of [^{3}H]choline influx

So far only the net uptake of [^{3}H]choline has been investigated. In view of the apparent differential monovalent cation sensitivity of net [^{3}H]choline uptake at high (102 μM) and low (0.48 μM) choline concentration, [^{3}H]choline influx was analyses at a range of different concentrations (Fig. 3). Lineweaver-Burk analyses of the influx, from 0 to 10 min of incubation, yielded points which were curvilinear in distribution for every condition studied. For further analyses the curves were resolved into two components and straight lines fitted by linear regression analyses. An example of the analyses is shown in Fig. 3.

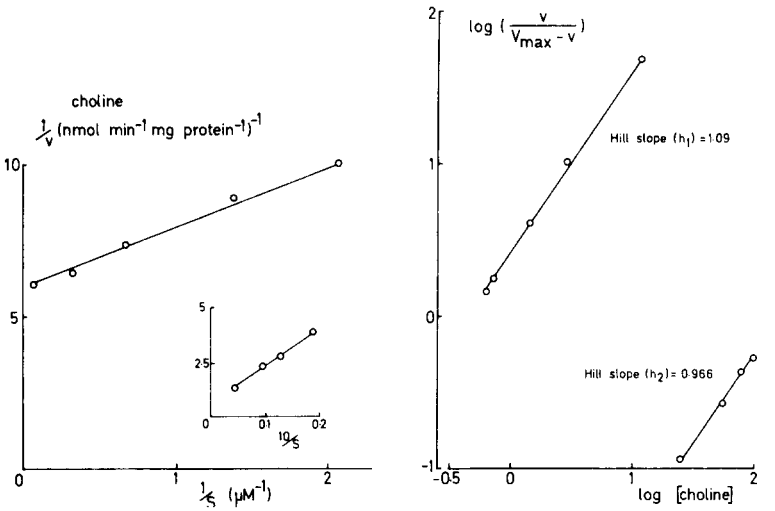

Fig. 3. The cation sensitivity of [^{3}H]choline influx at varying *trans*-choline concentrations. Example of the type of kinetic analysis employed. Synaptosome suspensions were incubated with varying concentrations of choline (0.48—102 μM) containing [^{3}H]choline. After 10 min of incubation, aliquots of the synaptosomal suspensions were removed and the [^{3}H]choline determined after filtration as described in Materials and Methods. Values were corrected for the small amount of binding of [^{3}H]choline to the filters found after lyses of the synaptosomes (Table III). Results were plotted by (a) Lineweaver-Burk method, and (b) as Hill Plots and are for [^{3}H]choline influx in the control (NaCl) condition. Straight lines were fitted by weighted linear regression analyses. The computed kinetic constants found for [^{3}H]choline when NaCl was replaced by Li^+, K^+, Rb^+ and Cs^+ are given in Table V.

The apparent kinetic constants for [^{3}H]choline influx were found from the linear intersection of these lines with the axes. A summary of the results of such analyses is shown in Table V. Replacement of Na^+ by other monovalent cations resulted in a decrease in the apparent maximal velocity of [^{3}H]choline influx for both the 'high' and 'low'-affinity systems with the exception of the apparent V (low affinity) for choline influx, in the presence of LiCl. In addition the apparent affinity constants for choline transport were increased when Na^+ was replaced by the other monovalent cations. When the kinetic data were

TABLE V

APPARENT KINETIC PARAMETERS FOR CHOLINE INFLUX

Incubation conditions are as described in Fig. 3. Apparent K_T and V values were derived from the double-reciprocal analyses of the influx versus the *cis*-choline concentration (Fig. 3). The Hill coefficients (h) were calculated from the slope of the linear regression lines (Fig. 3) using the equation [$\log (V/V-V) = h \log S - \log K_T$)], where the regression coefficients are given in parentheses.

Media	μM		pmol/min per mg protein		Hill coefficients	
	K_T (1)	K_T (2)	V_1	V_2	h_1	h_2
Na^+	0.33	208	167	1290	1.09 (0.997)	0.73 (0.976)
Li^+	1.65	387	44	2080	1.5 (0.997)	0.86 (0.814)
K^+	1.25	415	27	780	0.91 (0.979)	1.28 (0.99)
Rb^+	2.04	450	44	270	1.47 (0.983)	2.72 (0.979)

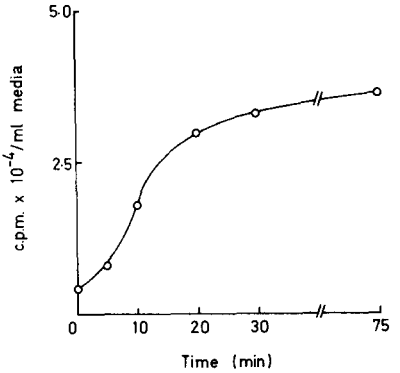

Fig. 4. Exodus of [^{3}H]choline from the synaptosomes. Synaptosomes were preloaded with [^{3}H]choline for 60 min in the control media at 22°C, thereafter deposited on membrane filters and [^{3}H]choline exodus recorded by taking small aliquots of the media at the chosen time points. Results are the means of quadruplicate samples in a typical experiment where S.D. are too small to be shown.

analysed by the use of Hill plots (Fig. 3, Table V) the slopes of both the high and low-affinity lines were approximately equal to 1 with the exception of the low-affinity transport in the presence of Rb^+, where the slope was somewhat greater.

Effects of monovalent cations and choline on [^{3}H]choline efflux

The exodus of [^{3}H]choline from the synaptosomes was studied (Fig. 4). The analyses of the exodus curves revealed three phases; a slower initial phase from 0 to 5 min (lag phase) followed by a more linear middle phase from 5 to 10 min and a much slower end phase (equilibrium) after 20 min of incubation. The linear portion of the exodus curve (5—10 min) was taken as representative of the [^{3}H]choline efflux. The addition of choline to the incubation medium resulted in a stimulation of [^{3}H]choline efflux in a dose-related fashion (Table VI), the efflux being highest at high *trans*-choline concentrations

TABLE VI

EFFECTS OF MONOVALENT CATIONS AND CHOLINE ON THE EFFLUX OF [^{3}H]CHOLINE

Experimental conditions were as described in Fig. 4. Results are the mean ± S.D. (n = 3) for the linear exodus over the 5—10 min time period and in some cases the content of [^{3}H]choline at 60 min incubation (equilibrium). —, not determined.

Condition	Concn. (μM)	Efflux (pmol/min per mg protein)	Equilibrium (% control)
Control	0.40	62 ± 5	100
Choline	0.48	103 ± 6	—
	2	157 ± 8	—
	50	175 ± 5	—
	100	201 ± 10	—
LiCl replaces NaCl		52 ± 4	78
KCl replaces NaCl		52 ± 6	86
RbCl replaces NaCl		85 ± 9	126
CsCl replaces NaCl		82 ± 10	140

(100 μM). Replacement of Na^+ by Cs^+ or Rb^+ also stimulated choline efflux but to a smaller degree than *trans*-choline, whereas the presence of Li^+ or K^+ resulted in a small decrease in the measured efflux. These effects of the cations on the efflux were reflected in similar changes in the equilibrium distribution of [^{3}H]choline (Table VI).

Discussion

The Na^+ and K^+ contents of the synaptosomes are smaller than the corresponding values of 477 μmol Na^+/g protein and 200—250 μmol K^+/g protein in mammalian synaptosomes, these differences probably being a manifestation of several factors including differences in the incubation conditions. The acetylcholine content of the synaptosomes is similar to that found in synaptosomes from squid head ganglia, but much larger than that of mammalian synaptosomes [22,26]. The results of the acetylcholine studies indicate that Sepia synaptosomes are capable of replenishing their stores of acetylcholine and in this respect they are similar to other isolated tissue preparations [4,26—29]. Several reports have described the inhibitory action of *N*-ethylmaleimide [2,5,30] and organic mercurials [2,31,32], on transport mechanisms in synaptosomes. These agents appear to affect several different transport mechanisms to a similar extent and in this respect their action appears to be non-selective.

On the basis of the close similarity of the recorded effects of CN^- + iodoacetate or ouabain, any claim of a primary energy-dependent transport of [^{3}H]-choline appears to be somewhat premature and it seems more probable that a collapse of the Na^+/K^+ gradients has some effects on [^{3}H]choline transport (see below). This point of view is strengthened by the results of the Na^+ replacement studies, where the total replacement of Na^+ by mannitol led to a similar reduction in [^{3}H]choline transport to that found with ouabain or CN^- + iodoacetate. Evidence that this reduction cannot be explained in terms of an inhibition in the further metabolism of [^{3}H]choline, comes from the [^{3}H]choline conversion studies which show that the transported molecule does not undergo appreciable metabolism, after traversing the membrane. Indeed, the transport of [^{3}H]choline into Sepia synaptosomes does not seem to be tightly coupled to acetylcholine synthesis, a conclusion which would agree with studies on the cholinergic tissue of *Torpedo* [33]. A different situation may persist, however, in mammalian synaptosomes [34].

It is unlikely that the inhibitory effects of the monovalent cations on [^{3}H]-choline uptake, are explicable in terms of a primary action on the ($Na^+ + K^+$)-ATPase or on energy metabolism, since the blockage of energy metabolism by ouabain caused at most a 30% reduction in [^{3}H]choline uptake, whereas the reduction found with the cations is much greater than this (Table IV). Hence, other explanations must be considered. At least as far as their interactions with water are concerned the alkali cations approximate to rigid non-polar spheres differing only in radius. The measured ionic radii are Li^+. 0.60 Å, Na^+ 0.95 Å, K^+ 1.33 Å, Rb^+ 1.48 Å, Cs^+ 1.69 Å [35] and the hydrated radii calculated from Stokes law are $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. Several possibilities can be envisaged which would explain the cation dependence of net [^{3}H]choline uptake, e.g. the cation may compete directly with choline for a limited number of

transport sites; a cation of defined radius may be required for the binding of choline at a separate site on the same protein; a pore in the membrane may require a cation of defined dimensions which may then exchange with choline and thereby allow choline to traverse the membrane (exchange pore model) or a monovalent cation of defined radius may make more sites available for choline transport across the limiting membrane.

The analyses of choline influx and efflux allow some choice as to which mechanism is more likely. The apparent kinetic parameters for choline influx are within the range of values found in other studies [2—4,23]. One of the major drawbacks in the comparison of parameters for transport in different types of nervous tissue preparations is the problem of the calculation of permeability constants which requires estimates of the membrane surface area and the internal volume of the tissue preparation. For the purpose of comparison it is worthwhile to obtain estimates of these two parameters. An estimate of 1.52 μl/mg protein from the volume of the synaptosomes can be calculated from the mean of the sodium chloride and potassium chloride volumes under the condition of metabolic inhibition (Table III) using the method of Marchbanks [36]. This apparent volume is somewhat smaller than the volume of mammalian synaptosomes of 3.2 μl/mg protein calculated from morphological [25] and biochemical analyses [36]. The surface area of the limiting membranes of the synaptosomes computed from the mean synaptosomal radius is 120 cm^2/mg protein, which is somewhat smaller than the value of 182 cm^2/mg protein found for mammalian synaptosomes [25]. A comparison of the ion concentrations and membrane potentials derived using this apparent volume with those found in other molluscan tissues gives some idea of the consistency of such calculations (Table VI). Despite the unavoidable inaccuracies in the estimation of the intrasynaptosomal volume, the computations still show a negative resting membrane potential and suggest that the synaptosomes are capable of concentrating K^+ and extruding Na^+ against their respective concentration gradients. This result is therefore in agreement with fluorometric studies [37], which show the presence of a resting membrane potential in synaptosomes.

Replacement of Na^+ by the other monovalent cations results in changes in

TABLE VII

COMPARISON OF APPARENT ION CONCENTRATION AND POTENTIALS IN *SEPIA* SYNAPTOSOMES WITH THOSE IN OTHER MOLLUSCAN TISSUES

Apparent ion concentrations in the synaptosomes were calculated from the ion contents in the control condition at the steady-state (Table IV) and the apparent volume of 1.5 μC/mg protein. Na^+, K^+ and Cl^- diffusion potentials (E_{Na}, E_K and E_{Cl}) (mV) were computed from the Nernst equation and the apparent transmembrane potential (E_m) (mV) was estimated from the Hodgkin et al. equation (Ref. 22). Values for the potentials of snail neurone and *Sepia* axon are the measured potentials. $[Na_t]$, $[K_t]$ and $[Cl_t^-]$ are in mM.

Tissue	$[Na_i^+]$	$[K_i^+]$	$[Cl_i^-]$	E_{Na}	E_K	E_{Cl}	E_m	Ref.
Sepia axon	120	250	149	+25	—61	—23	—60	41,42
Snail neurone	—	93	—	—	—43	—	—41	43
Synaptosomes	172	62	280	+16	—32	—19	—16	

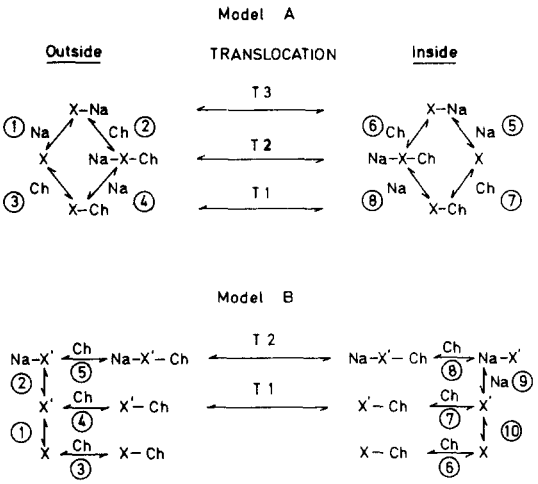

Fig. 5. Model A. Na^+ increases the rate of translocation of the carrier (X)-choline (Ch) complex across the synaptic limiting membrane. The carrier (X) can combine with either (or both) Na^+ and choline. However, the coefficient of translocation (permeability) for the Na^+-X-choline complex (T_2) is greater than the X-choline complex (T_1). Model B. Na^+ increases the total number of active sites available for choline transport. An inactive form of the carrier (X) is in equilibrium with its active form (X'). Choline has the same binding affinity for the two carrier states ($K_3 = K_4$) but only X' can translocate choline across the neuronal membrane.

the apparent V and K_m values of the transport system for choline. In this case it must be stressed that the apparent K_m value is a particular combination of kinetic constants of several different reactions, such as those shown in Fig. 5 (see also [38]). Within the experimental framework used it is impossible to delineate the contributions made by the different processes. In accord with other Na^+-dependent transport mechanisms [39] the results are suggestive of an ion-linked cotransport of choline across the presynaptic plasma membrane. In view of the relatively small effects of the metabolic inhibitors on choline transport, the results are consistent with transport of the secondary active type. There are at least two models which have been proposed for the mechanism of secondary transport [40]. In general these involve an 'affinity' effect or a 'velocity' effect. In the former case the binding of one solute to the mechanism raises the affinity of the carrier for the other solute and vice versa. This effect alone provides energetic coupling. a 'velocity' effect results when the translocation rate of the ternary complex across the membrane is faster then either of the two possible binary complexes. This also affords energy coupling in the absence of affinity effects. In the present case the replacement studies suggest that a combination of these effects exists, although as with the interpretation of all kinetic data from transport studies of this type, a major complicating factor is the electrical potential across the plasma membrane. In the case where the driver solute is a cation and where the organic solute has a net positive charge, some of the resulting complexes with the carrier may be electrically charged and will be susceptible thereby to the driving force of the electrical field. In the case of choline transport, this effect may be, however, less predominant than at first sight, since there is also evidence for an involvement of Cl^- in choline transport [41]. This may mean that positive charges on the

choline transport mechanism are neutralised to some extent by Cl^-. In general if must be conceded that certain functional advantages would be conferred by coupling the transport of choline to Na^+, for example, this would provide a means of coordinating choline flux to the changing demands of nervous tissue for acetylcholine.

Despite all the imponderables of the experimental system, an appraisal of the available data on choline transport allows some suggestions as to the possible mechanisms for the translocation across the presynaptic limiting membrane (Fig. 5). Both hypothetical models shown involve a one-to-one interaction of choline (Ch) with some form of carrier mechanism (X) as suggested by the Hill plots. The number of Na^+ involved in the mechanism remains to be established. In one model, Na^+ increases the rate of translocation of the carrier-choline complex (model A), in the other Na^+ increases the total number of active sites available for choline binding (model B). In both models it is conceivable that the translocation of the choline carrier complex, without the necessary involvement of Na^+, becomes predominant at high choline concentrations or of course when Na^+ is absent, thus explaining: the ability of choline to be transported when mannitol replaces Na^+; the differences in the monovalent cation sensitivity of the high and low-affinity systems and the ability of *trans*-choline to stimulate the efflux of choline. A more exact appraisal of choline transport will ultimately rest upon the isolation of the components of the transport system. For this purpose nerve ending fractions which are potentially highly enriched in cholinergic membranes, such as those from *Torpedo* and cephalopod, are likely to be of major importance.

References

1 Marchbanks, R.M. (1968) Biochem. J. 110, 523—541
2 Yamamura, H.I. and Snyder, S.H. (1973) J. Neurochem. 21, 1355—1374
3 Haga, T. and Noda, H. (1973) Biochim. Biophys. Acta 291, 564—575
4 Dowdall, M.J. and Simon, E.J. (1973) J. Neurochem. 21, 969—982
5 Holden, J.T., Rossier, J., Beaujouan, J.C., Guyenet, P. and Glowinski, J. (1975) Mol. Pharmacol. 11, 19—27
6 Eisenstadt, M.L., Treistman, S.N. and Schwartz, J.H. (1975) J. Gen. Physiol. 65, 275—291
7 Hodgkin, A.L. and Martin, K. (1965) J. Physiol. 179, 26—27P
8 Martin, K. (1972) J. Physiol. 224, 207—230
9 Diamond, I. and Milfay, D. (1972) J. Neurochem. 19, 1899—1909
10 Kuhar, M.J., Sethy, V., Roth, H.R. and Aghajanian, G.K. (1973) J. Neurochem. 20, 581—593
11 Ansell, G.B. and Spanner, S. (1967) J. Neurochem. 14, 873—885
12 Campbell, C.W.B. and Dowdall, M.J. (1975) Exp. Brain Res. 24, p7
13 Campbell, C.W.B. (1976) 10th. I.U.B., Hamburg, p. 563
14 Dowdall, M.J. and Whittaker, V.P. (1973) J. Neurochem. 20, 921—935
15 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
16 Racker, E. (1950) Biochim. Biophys. Acta 4, 211—214
17 Whittaker, V.P. and Barker, L.A. (1972) in Methods in Neurochemistry (Fried, R., ed.), Vol. 2, pp. 1—52, Marcell Dekker, New York
18 Fonnum, F. (1975) J. Neurochem. 24, 407—409
19 Campbell, C.W.B. (1978) Biochem. Soc. Trans. 6, 389—391
20 Baudhuin, P. and Berthet, J. (1967) J. Cell Biol. 35, 631—648
21 Marchbanks, R.M. and Campbell, C.W.B. (1976) J. Neurochem. 26, 973—980
22 Campbell, C.W.B. (1976) Brain Res. 101, 594—599
23 Campbell, C.W.B. (1978) Neurosci. Lett. 8, 341—346
24 Jones, D.G. (1967) J. Cell Sci. 2, 573—586
25 Clementi, F., Whittaker, V.P. and Sheridan, M.N. (1966) Z. Zellforsch. 72, 126—138

26 Bradford, H.F. (1970) Brain Res. 19, 239—247
27 Collier, B., Poon, P. and Salehmoghaddam, B. (1972) J. Neurochem. 19, 51—60
28 Bhatnagar, S.P. and MacIntosh, F.C. (1967) Can. J. Physiol. Pharmacol. 45, 249—268
29 Bourdois, P.S. and Szerb, J.C. (1972) J. Neurochem. 19, 1189—1193
30 Diamond, I. and Kennedy, E.P. (1969) J. Biol. Chem. 244, 3258—3263
31 Haga, T. (1971) J. Neurochem. 18, 781—798
32 Osborne, R.H. and Bradford, H.F. (1975) J. Neurochem. 25, 35—41
33 Dowdall, M.J., Fox, G., Wächtler, K., Whittaker, V.P. and Zimmermann, H. (1976) Cold Spring Harbour Symp., Vol. XL, pp. 65—81
34 Barker, L.A. and Mittag, T.W. (1975) J. Pharmacol. Exp. Ther. 192, 86—92
35 Pauling, L. (1948) in The Nature of the Chemical Bond, Cornell University Press
36 Marchbanks, R.M. (1975) J. Neurochem. 25, 463—470
37 Blaustein, M.P. and Goldring, J.M. (1975) J. Physiol. 247, 589—615
38 Stein, W.D. (1967) Movement of Molecules across Cell Membranes, p. 177-ff, Academic Press
39 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637—718
40 Heinz, E., Geck, P. and Wilbrandt, W. (1972) Biochim. Biophys. Acta 255, 442—461
41 Kuhar, M.J. and Zarbin, M.A. (1978) J. Neurochem. 31, 251—256