Br. J. Pharmac (1983), **78**, 111–116

Storage of biogenic amines in guinea-pig brain synaptosomes: influence of proton gradient and membrane potential

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1 The effects of K^+, NaCN and the ionophores monensin, nonactin and carbonyl-cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP) on the contents of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT), [^{3}H]-dopamine and [^{3}H]-noradrenaline ([^{3}H]-NA) in guinea-pig synaptosomes preloaded with these amines were measured.

2 In the presence of Ca^{2+}, K^+ markedly reduced the amine content of the synaptosomes, indicating an acceleration of spontaneous amine release. In the absence of Ca^{2+}, K^+ had much less effect.

3 Monensin, nonactin and FCCP caused a release of all the three labelled amines. This release was considerably faster and more marked than that induced by K^+ and showed no dependence on Ca^{2+}. The ionophores did not release lactate-dehydrogenase from synaptosomes.

4 NaCN, a blocker of oxidative energy production, did not enhance the spontaneous release of [^{3}H]-5-HT nor did it influence the monensin-induced release of [^{3}H]-5-HT.

5 It is concluded that (a) the intragranular storage of 5-HT, dopamine and NA is dependent on the maintenance of a pH-gradient across the granular membrane as well as on the granular membrane potential; (b) the ionophores cause a non-exocytotic release of granular amines, and (c) blood platelets are partial models for aminergic brain neurones as far as intragranular amine storage is concerned.

Introduction

The mechanism of accumulation of biogenic amines in the storage organelles of cerebral nerve terminals has not been fully elucidated. Experiments with isolated storage vesicles of brain have indicated that, as in other amine storage organelles (e.g. of adrenal medulla and blood platelets), a proton gradient across the granular membrane (pH inside acid) seems to be essential (Toll & Howard, 1978). The influence of the granular membrane potential is not clear.

In the present work the roles of the granular pH-gradient and of the membrane potential for the storage of biogenic amines in brain have been investigated in cerebral synaptosomes (pinched-off nerve endings) using different ionophores. Synaptosomes were chosen since membranes and contents of storage organelles kept in their natural environment (i.e. within the nerve ending) are probably better preserved than those of isolated vesicles. The following ionophores were used: carbonyl-cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP; transporting protons), nonactin (transporting K^+ and Na^+) and monensin (catalysing electroneutral exchange of H^+, Na^+ and K^+) (Pressman, 1976). The results are compared with those previously obtained in blood platelets, which have been proposed as partial models for monoaminergic neurones (Pletscher, 1969; Sneddon, 1973).

Methods

Experimental procedure

Synaptosomes were prepared from the mesencephalon plus hypothalamus of guinea-pigs according to the method of Whittaker & Barber (1972) as modified by Peyer, Pletscher & Affolter (1982). Portions of synaptosomes corresponding to 1 mg protein (determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard) were diluted to a final volume of 15 ml with Ca-buffer containing (mM): NaCl 118, HEPES 20, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, $CaCl_2$ 2.53, glucose 1.11, ascorbic acid 1.14, disodium edetate

0007-1188/83/010111-06-$01.00

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(Na_2EDTA) 0.133 and 12.5 μM nialamide (final pH 7.4 at 37°C). In other experiments the synaptosomes were prepared and diluted in the above buffer with the $CaCl_2$ replaced by 2 mM EGTA (EGTA-buffer). In some experiments 35 mM Na^+ was replaced by K^+ (final concentration 40 mM).

The granular amines were labelled by incubation of the synaptosomes with 100 nM [^{3}H]-5-HT, 50 nM [^{3}H]-dopamine or 50 nM [^{3}H]-NA for 20 min at 37°C. Part of the synaptosomes were preincubated with 2 μM reserpine (Peyer *et al.*, 1982) for 60 min at 37°C prior to the incubation with the labelled amines. Nonspecific binding in each case was determined by carrying out the experiment with synaptosomes that had been incubated with labelled amines in the presence of a 1000 fold excess of the corresponding unlabelled amine. The suspensions were divided into portions of 1.25 ml (corresponding to 83 μg synaptosomal protein) and the synaptosomes isolated by centrifugation at 4300 *g* for 10 min at 4°C. To start the release experiments the preloaded synaptosomes were resuspended in 5 ml of prewarmed Ca- or EGTA-buffer (37°C), to which the different substances had been added. In some of the experiments mitochondrial energy transduction was inhibited by the addition of 1 mM NaCN. At suitable time intervals, 1 ml-portions of the suspension were withdrawn, filtered on Whatman GF/C filters under vacuum and washed twice with 4 ml ice-cold buffer containing (mM): NaCl 133, KCl 7, Tris 10 (final pH 7.4 at 4°C). Filters were put into counting vials, which were vigorously shaken after addition of 10 ml Quickszint 212. Radioactivity was counted in a Kontron MR 300 DPM counter and compared with the initial radioactivity, determined by resuspending samples of preloaded synaptosomes in ice-cold medium and filtering immediately. For the time course experiments the ionophores were used in concentrations which induced a [^{3}H]-5-HT-release of 85–90% in 10 min.

To determine the release of lactate-dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) into the incubation medium, portions of synaptosomes corresponding to 0.5 mg protein were incubated in the same way as in the amine-release experiments, with or without the different ionophores. After 10 min the suspension was centrifuged at 8000 *g* and 4°C for 5 min and the supernatant separated. Lactate-dehydrogenase was measured in the synaptosomal pellet (lysed with 150 μl 1% Triton-X 100) and in the supernatant by a spectrophotometric method (Kornberg, 1955) at 340 nm and 25°C. Samples of 50 μl synaptosomal lysates and supernatants were added to 1 ml medium containing 2 mM pyruvate and 100 mM sodium phosphate buffer (pH 7.5). The reaction was started by adding 0.25 mg Na_2NADH in 1 ml. Blanks obtained by incubation without pyruvate were subtracted.

In order to control whether [^{3}H]-dopamine was converted to [^{3}H]-NA, synaptosomes were loaded

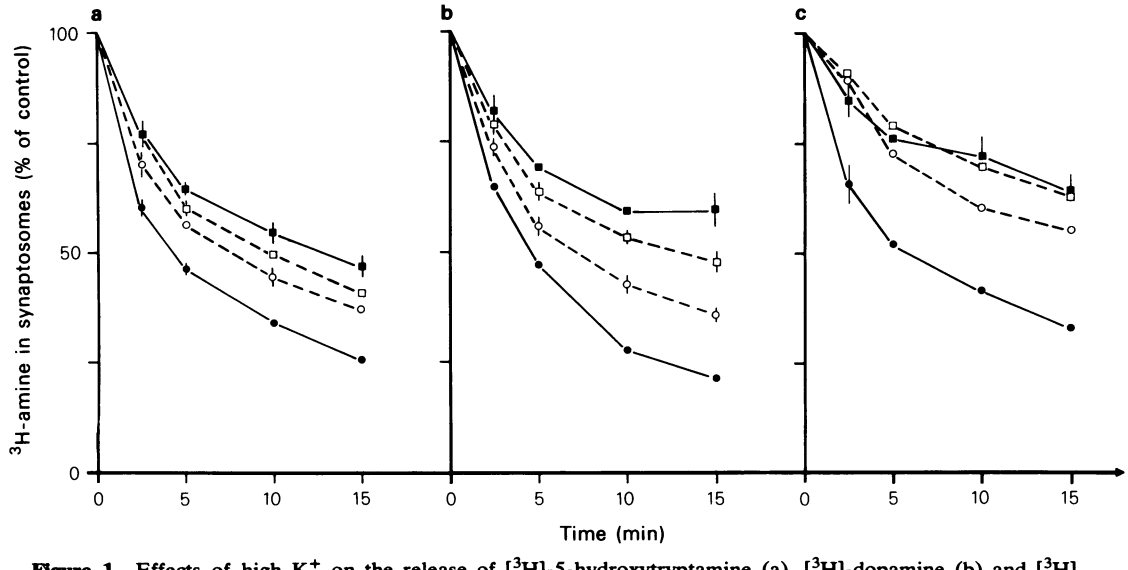

Figure 1 Effects of high K^+ on the release of [^{3}H]-5-hydroxytryptamine (a), [^{3}H]-dopamine (b) and [^{3}H]-noradrenaline (c). The ^{3}H-amine content of the synaptosomes is given as % of that in synaptosomes resuspended in ice-cold medium and filtered immediately (averages with s.e.mean of at least 6 experiments). Nonspecific radioactivity was deduced. Controls: squares (□, ■); increased K^+: circles (○, ●); Ca^{2+}-buffer: straight lines, closed symbols; EGTA-buffer: broken lines, open symbols. The values with K^+ in Ca^{2+}-medium were significantly different from those of the corresponding controls. In the figures s.e.mean is only indicated when greater than 2%.

with [^{3}H]-dopamine and incubated for 30 min as described above. Then 15 μM (final concentration) monensin was added to release the intracellular catecholamines and the mixture incubated for a further 5 min. The supernatant of the synaptosomal suspension as well as standard solutions containing [^{3}H]-dopamine and [^{3}H]-NA were then mixed with unlabelled (carrier) dopamine and NA and submitted to thin layer chromatography on polygram cel 400 UV 254 (Machery-Nagel & Co., Düren, West Germany) using butanol/H_2O/acetic acid (12:5:3 v/v) as solvent. The bands were extracted with 1 M HCl, supplemented with scintillator and the radioactivity measured in a scintillation counter. Over 85% of the radioactivity added to the synaptosomal suspension was found to have the same R_F value as [^{3}H]-dopamine and less than 7% had the same R_F-value as [^{3}H]-NA.

Calculations

The counted values from the labelled samples were corrected for quenching and for counting efficiency by the ^{3}H-programme of the scintillation counter. Specific radioactivity was calculated as total radioactivity on the filter minus the nonspecific value. Results are then expressed as percentages taking the specific radioactivity of the sample resuspended in ice-cold medium as 100%. Statistical analyses were performed with the distribution-free Wilcoxon rank sum test ($\alpha < 0.05$, one-sided).

Materials

5-Hydroxy [G-^{3}H] tryptamine creatinine sulphate (specific activity 6.5×10^{14} Bq/mol), (−)-[7,8-^{3}H]-noradrenaline (specific activity 1.33×10^{15} Bq/mol) and [7,8-^{3}H]-dopamine (specific activity 1.74×10^{15} Bq/mol) were obtained from Amersham International Limited. Nonactin was purchased from Fluka AG (Buchs, Switzerland), monensin from Calbiochem AG (Luzern, Switzerland), carbonyl-cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP) from Boehringer Mannheim AG (Cham, Switzerland), nialamide from Sigma (Bender & Hobein AG, Zürich, Switzerland) and Quickszint 212 from Radium Chemie AG (Teufen, Switzerland). The rest of the chemicals was available from commercial sources.

Results

Absolute values

At the beginning of the experiments (zero time) the synaptosomes contained 162 ± 6 pmol [^{3}H]-5-HT, 112 ± 8 pmol [^{3}H]-dopamine and 61 ± 5 pmol [^{3}H]-NA per mg protein (mean with standard error of 5 experiments each). Reserpine-treated synaptosomes accumulated 64 ± 6 pmol [^{3}H]-5-HT per mg protein.

Spontaneous release, role of Ca^{2+}

Synaptosomes incubated in EGTA-buffer showed a time-dependent decrease of [^{3}H]-5-HT, [^{3}H]-dopamine and [^{3}H]-NA (Figure 1), indicating a spontaneous release of these amines. The release was similar in magnitude for the three amines and had an exponential time course. Replacement of EGTA by Ca^{2+} (2.53 mM) did not significantly influence the spontaneous release of the amines (Figure 1).

Effect of high K^+

When 35 mM Na^+ of the Ca^{2+}-containing buffer was replaced by K^+ (final concentration 40 mM) the release of the amines was markedly enhanced (Figure 1). After 15 min the contents of [^{3}H]-5-HT, [^{3}H]-dopamine and [^{3}H]-NA were 25, 21 and 33% (of the total amine content at time zero) respectively, compared with 46, 59 and 64% in the normal Ca^{2+}-buffer.

In Ca^{2+}-free buffer K^+ (40 mM) decreased the content of [^{3}H]-5-HT, [^{3}H]-dopamine and [^{3}H]-NA from 37, 48 and 62% (controls) to only 35, 36 and 55% respectively (Figure 1, decrease of [^{3}H]-5-HT and [^{3}H]-NA content not significant).

Effect of ionophores, dose-response

In buffer devoid of Ca^{2+} (containing EGTA) rising concentrations of the ionophores caused an increasing release of [^{3}H]-5-HT. After an incubation of 10 min, the releases induced by FCCP, nonactin and monensin were approximately 90% at concentrations of the ionophores of 16, 10 and 4.5 μM respectively (Figure 2). [^{3}H]-dopamine and [^{3}H]-NA behaved similarily to [^{3}H]-5-HT.

Effect of ionophores, time course

In buffer devoid of Ca^{2+}, FCCP, monensin and nonactin caused a rapid release of the amines (Figure 3), which reached 80–90% after 10 min and whose time course was not different from that of the amine release caused by the ionophores in buffer containing Ca^{2+}.

Lactate-dehydrogenase

During incubation for 10 min of synaptosomes with and without ionophores in concentrations used in the amine-release experiments, over 90% of lactate-

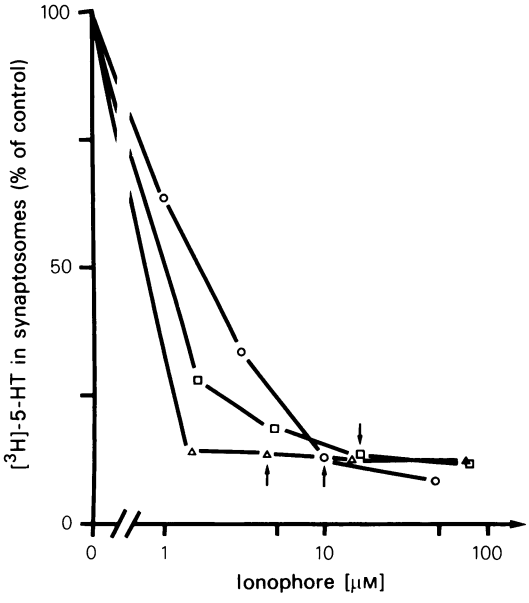

Figure 2 Dependence of the [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) release on the ionophore concentration. Synaptosomes were incubated for 10 min in Ca-buffer alone (controls) or Ca-buffer containing monensin (△), nonactin (○) or FCCP (□) in the concentrations indicated on the horizontal-axis (logarithmic scale). The [^{3}H]-5-HT-content is given as % of that of controls (averages of duplicates). The arrows indicate the concentrations chosen for the time course experiments.

dehydrogenase activity remained in the synaptosomal pellet. The amounts of the enzyme appearing in the supernatant of preparations incubated with the ionophores were not significantly different from those in the controls (Table 1).

Effect of NaCN

NaCN (10^{-3}M) added to synaptosomes suspended in buffer devoid of Ca^{2+} (containing EGTA) did not significantly change the spontaneous release of [^{3}H]-5-HT. Also, the monensin-induced release of the amine was not significantly influenced by NaCN.

Discussion

There is good evidence (Schwartz, 1981) that a large part of the radiolabelled amines taken up by the synaptosomes is accumulated in the storage granules. This has been shown by determinations of the subcellular distribution of labelled amines in brain homogenates (Whittaker, 1970). Furthermore, in the present and previous experiments (Peyer *et al.*, 1982) reserpine and reserpine-like drugs (benzoquinolizine derivatives), which are known to interfere specifically with granular amine storage, led to a marked inhibition of accumulation and release of labelled amines in synaptosomes. In addition, the amine release caused by K^+-induced depolarization of the synaptosomal plasma membrane was dependent on Ca^{2+}, confirm-

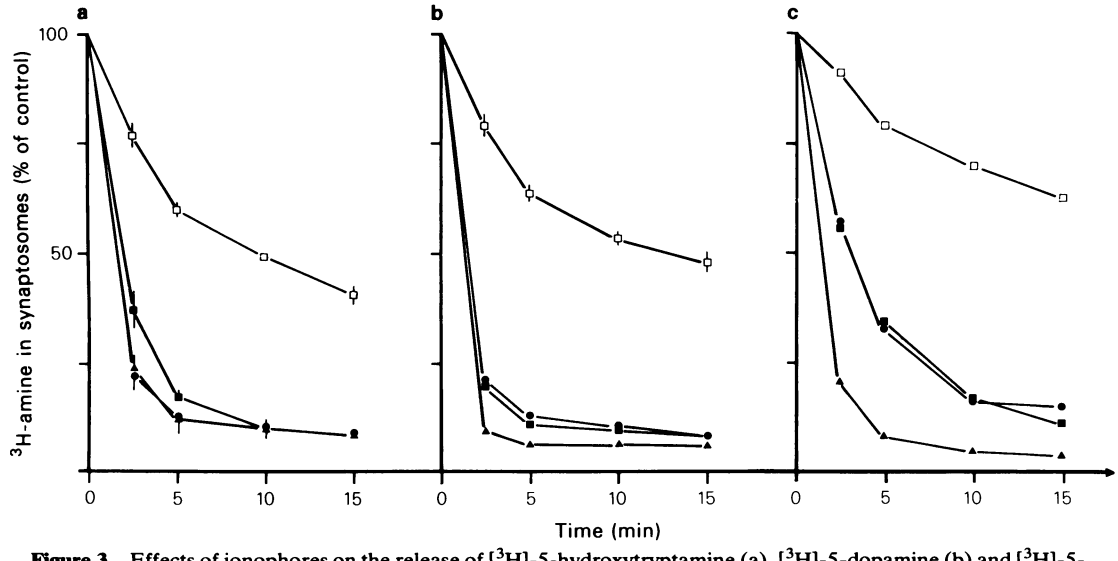

Figure 3 Effects of ionophores on the release of [^{3}H]-5-hydroxytryptamine (a), [^{3}H]-5-dopamine (b) and [^{3}H]-5-noradrenaline (c) in EGTA-buffer; time course. The ^{3}H-amine content of the synaptosomes is given as % of that in synaptosomes resuspended in ice-cold medium and filtered immediately (averages with s.e.mean of at least 6 experiments). Releasing agents: (□) none (controls); (▲) monensin; (●) nonactin; (■) FCCP. S.e.mean is only indicated when greater than 2%.

Table 1 Distribution of lactate-dehydrogenase

Addition	*Incubation*	*Enzyme activity*	
		Synaptosomes	*Supernatant*
	(min.)	(%)	(%)
None	0	95±3	4.8±2.8
None	10	94±4	6.1±4.0
Monensin	10	92±4	8.0±4.2
Nonactin	10	92±4	7.8±4.2
FCCP	10	95±4	4.4±2.6

The activity of lactate-dehydrogenase is given as % of the activity in the lysed synaptosomal pellet plus that in the corresponding supernatant (means±s.e.mean of 3 independent experiments). The ionophores were added in the same amount per mg synaptosomal protein as in the amine-release experiments.

ing earlier findings of West & Fillenz (1981). This is in agreement with a granular localization of the released amines, since it indicates that the amines are released by exocytosis of granular contents, which has been shown to be Ca^{2+}-dependent (Katz & Miledi, 1967).

All three ionophores (FCCP, monensin and nonactin) caused a Ca^{2+}-independent release of the amines from brain synaptosomes. In preliminary experiments the ionophores also had a similar action on [^{3}H]-dopamine in striatal synaptosomes. The release caused by the ionophores was much faster than that induced by K^+ in the presence of Ca^{2+}. These findings indicate that exocytosis due to depolarization of the plasma membrane was probably not a major factor. Also, the amine-releasing effect of the ionophores was not due to lysis of the synaptosomes, because none of the three ionophores caused a release of the cytoplasmatic enzyme, lactate-dehydrogenase. Therefore a major action of the ionophores at the plasma membrane seems rather unlikely, although some alteration at this level cannot be excluded.

It has been reported that monensin, nonactin and FCCP induce at least a transient disturbance of oxidative phosphorylation in mitochondria (Pressman, 1976). However, our experiments show that this mechanism is unlikely to be a major factor in the amine-releasing action of the ionophores. Thus, NaCN, which blocks oxidative energy production, did not enhance the spontaneous [^{3}H]-5-HT-release.

Therefore, the ionophores seem to act mainly at the level of the amine storage organelles where they cause a non-exocytotic amine release. This result is in agreement with findings in other amine storage systems, e.g. of blood platelets (Affolter & Pletscher, 1982) and of adrenal medullary granules (Johnson, Pfister, Carty & Scarpa, 1979).

The mode of action of ionophores in interfering with intragranular amine storage has been mainly investigated in peripheral systems, whereas much less is known about their action in synaptosomes. The concentrations of Na^+ and K^+ in the cytoplasm of nerves are approximately 14 and 120 mM respectively (Schadé & Ford, 1973), but their intragranular concentrations are not known. However, the action of FCCP, which transports only protons, does not depend on Na^+- or K^+-gradients. This ionophore, which caused a marked amine release, induces a fast collapse of the membrane potential followed by a slower reduction of the proton gradient (Carty, Johnson & Scarpa, 1981). Therefore the amine-releasing effect of FCCP seems to be connected with a change of the granular membrane potential and/or of the proton gradient across the granular membrane. In contrast, the actions of monensin and nonactin are influenced by Na^+- and K^+-gradients. However, it can be assumed that the concentrations of Na^+ and K^+ in the granules are together not greater than the concentration in the cytoplasm, since this would raise osmotic pressure, leading to damage of the granules. Therefore a downhill gradient of both ions from inside to outside the granules is unlikely. Assuming this, monensin, which catalyses an electroneutral exchange of H^+, Na^+ and K^+, could cause a rapid reduction of the H^+-gradient normally present across the granular membrane, without directly changing granular membrane potential. On the other hand, nonactin, which transports Na^+ and K^+, probably affects the granular membrane potential without influencing the proton gradient. Therefore, since monensin and nonactin have an amine-releasing effect, the intragranular storage of amines is likely to depend on both the proton gradient across the granular membrane and the granular membrane potential.

The granular amine storage in brain synaptosomes seems to be similar in certain respects to that in blood platelets. For instance, in both systems this storage probably depends to a considerable degree on a proton gradient across the granular membrane. However, intracellular storage in synaptosomes (in contrast to that in platelets) also appears to be mar-

kedly dependent on the granular membrane potential. In addition, synaptosomes but not platelets showed a K^+-induced, Ca^{2+}-dependent (probably exocytotic) amine release. It is not yet known whether intragranular interaction of amines with ATP plays an important role in amine storage in synaptosomal granules, as it does in storage organelles of platelets.

The authors thank Dr H. Döbeli for technical advice and Miss Fabrizia Ferracin for skillful technical assistance.

References

AFFOLTER, H. & PLETSCHER, A. (1982). Storage of biogenic amines in intact blood platelets of man: dependence on a proton gradient. *J. mol. Pharmac.*, **22**, 94–98.

CARTY, S.E., JOHNSON, R.G. & SCARPA, A.(1981). Serotonin transport in isolated platelet granules. *J. biol. Chem.*, **256**, 11244–11250.

JOHNSON, R.G., PFISTER, D., CARTY, S.E. & SCARPA, A. (1979). Biological amine transport in chromaffin ghosts. *J. biol. Chem.*, **254**, 10963–10972.

KATZ, B. & MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. *J. Physiol.*, **189**, 535–544.

KORNBERG, A. (1955). Lactic dehydrogenase assay. *Meth. Enzymol.*, **1**, 441.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.T. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

PEYER, M., PLETSCHER, A. & AFFOLTER, H. (1982). Drug-induced release of biogenic amines from synaptosomes and blood platelets of guinea pigs. *Neuropharmac.*, **21**, 831–838.

PLETSCHER, A. (1969). Metabolism, transfer and storage of 5-hydroxytryptamine in blood platelets. *Br. J. Pharmac. Chemother.*, **32**, 1–16.

PRESSMAN, B.C. (1976). Biological applications of ionophores. *A. Rev. Biochem.*, **45** 501–530.

SCHADÉ, J.P. & FORD, D.H. (1973). In *Basic Neurology*. 161. New York/Amsterdam/Oxford: Elsevier.

SCHWARTZ, J.H. (1981). Vesicles store and release. In *Principles of Neural Science*, ed. Kandel, E.R. & Schwartz, J.H. 113–116. New York/Amsterdam/Oxford: Elsevier.

SNEDDON, J.M. (1973). Blood platelets as a model for monoamine-containing neurons. In *Neurobiology*, ed. Kerkut, G.A. & Phillis, J.W., Vol. 1, part 2, 151–198. Oxford: Pergamon.

TOLL, L. & HOWARD, B.D. (1978). Role of Mg^{2+}-ATPase and a pH-gradient in the storage of catecholamines in synaptic vesicles. *Biochemistry*, **17**, 2517–2523.

WEST, D.P. & FILLENZ, M. (1981). Control of noradrenaline release from hippocampal synaptosomes. *J. Neurochem.*, **37**, 1052–1053.

WHITTAKER, V.P. (1970). The investigation of synaptic function by means of subcellular fractionation techniques. In *The Neurosciences: Second Study Program*, ed. Schmitt, F.O. 761–768. New York: Rockefeller University Press.

WHITTAKER, V.P. & BARKER, L.A. (1972). The subcellular fractionation of brain tissue with reference to the preparation of synaptosomes and their component organelles. In *Methods of Neurochemistry*, ed. Fried, R. 1–52. New York: M. Dekker Inc.

(Received June 3, 1982. Revised September 14, 1982.)