Biochemical Pharmacology, Vol. 28, pp. 2091–2094.

0006-2952/79/0701-2091 $02.00/0

Inhibition by reserpine of calcium-dependent release of [^{3}H]norepinephrine from synaptosomes depolarized with potassium or veratridine*

(*Received* 4 *August* 1978; *accepted* 11 *December* 1978)

There is a continuing controversy in neurobiology as to whether neurotransmitters are released directly from synaptic vesicles by exocytosis, or by some other mechanism involving transmitter in the cytoplasm or bound to nerve terminal membranes. On the one hand, the unquestioned presence of acetylcholine [1] and norepinephrine (NE) [2, 3] in synaptic vesicles, the demonstrated fusion of cholinergic vesicles with pre-synaptic membranes at specific locations [4], and the quantal nature of post-synaptic responses [5, 6] suggest that release is indeed an exocytotic process. On the other hand, there is electrophysiological evidence for a rapid diminution in the release of acetylcholine despite the output of only 1–2 per cent of total stores [7], and considerable evidence from isotopic labeling experiments that the pool of stored acetylcholine or NE from which release occurs is not fully equivalent to either vesicular or non-vesicular stores [8–10]. Many investigators, therefore, have come to the conclusion that vesicles near the pre-synaptic membrane represent a spatially and/or temporally separable "retail" pool of transmitter available for release, while others suspect that vesicles replenish hypothetical "release sites", presumably via the cytoplasm.

In order to obtain further information concerning the source of released NE, several groups of investigators have made use of reserpine, which prevents the uptake and/or storage of catecholamines in vesicles, and of monoamine oxidase (MAO) inhibitors which block the metabolism of NE in the neuroplasm. Reserpine-treated tissues exposed to NE and an MAO inhibitor show uptake and retention of the amine by fluorescence microscopy [11, 12] and by biochemical assay [2, 13, 14], but little binding within vesicles, as demonstrated by counts of small granular vesicles [11] or by subcellular fractionation [2, 13]. It has been shown for the heart [15], iris [16, 17] and vas deferens [14] that such cytoplasmic transmitter does not support normal neurotransmission and the production of usual post-synaptic cell responses. For the iris and vas deferens, it has been shown, in addition, that the spontaneous release of [^{3}H]NE is high in relation to total store size, but that nerve stimulation is proportionally much less effective in releasing the retained amine in the presence than in the absence of reserpine [2, 14, 17]. We have chosen to continue this approach with synaptosomes because of our ability to control, compare and rapidly change such variables as the concentrations of calcium and potassium ions and of drugs surrounding these nerve terminals. A variety of laboratories have used synaptosomes to demonstrate high affinity uptake and Ca^{2+}-dependent, potassium-stimulated release of NE [18]. Uptake characteristics were similar in synaptosomal fraction regardless of whether the labeling occurred before or after isolation, which suggests that uptake *in vitro* can be used as a relevent measure of uptake *in situ*. Release of endogenous and exogenously applied NE from synaptosomes is stimulated by chemical depolarization and demonstrates most of the known characteristics of physiologically relevant release processes *in situ* including differential divalent cation sensitivity [19].

Male Sprague–Dawley rats, weighing 200–300 g, were given either reserpine (Serpasil, CIBA Pharmaceutical Co., Summit, NJ; 5 mg/kg, intraperitoneally) or an equivalent volume of the same parenteral solution without the drug (Serpasil placebo) 18 hr before each experiment. The animals were guillotined, and crude synaptosome fractions were prepared from forebrain regions by the method of Cotman and Matthews [20]. In brief, homogenates in ice-cold 0.32 M sucrose were centrifuged at 200 *g* for 15 min to remove large particles, and again at 15,000 *g* for 12 min to collect synaptosomes. These were resuspended in ice-cold sucrose at a protein concentration of about 5 mg/ml. The exact amount of protein was determined [21] after each experiment.

Uptake of [^{3}H]*NE.* Unless noted otherwise, 0.1-ml aliquots of synaptosomes were diluted with 9 vol. of buffer at 37°, containing (prior to dilution) 150 mM NaCl, 6.2 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 1 mM ascorbic acid, 20 mM *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid, sufficient Tris base to adjust the pH to 7.4, 1 μM pargyline to inhibit MAO, and 0.2 μM [^{3}H]NE (13 Ci/mmole; New England Nuclear Corp., Boston, MA), and were incubated for 4 min at 37°. The buffer was bubbled for 30 min with 95% O_2–5% CO_2 before used but not during incubations. Each sample was then transferred to a Whatman (Clifton, NJ) GF/A glass fiber filter held on a suction manifold, and washed three times with 2 ml of undiluted buffer at 37° without NE. Total radioactivity on filters was assessed after dissolving particles in 2 ml of 1% sodium dodecyl sulfate in 20 mM ethylenediaminetetraacetate, by liquid scintillation spectrometry in a toluene–Triton X-100 phosphor [22].

The proportion of radioactivity remaining as [^{3}H]NE was determined by subjecting lysates of washed synaptosomes to thin-layer chromatography (Eastman, Rochester, NJ, Chromogram cellulace sheets) in 1-butanol–methanol–1 N formic acid (60:20:20). More than 90 per cent of the radioactivity retained in synaptosomes from control and reserpine-treated animals, after incubation in 0.2 to 2 μM [^{3}H]NE for 1–10 min, co-chromatographed with authentic NE.

As in earlier experiments [23], the uptake with retention of [^{3}H]NE in control synaptosomes was saturable and both Na^+- and temperature-dependent: at 37° it amounted to 12 ± 0.6 nmoles/mg of protein; at 4° to 0.2 ± 0.4 nmole/mg; and in Na^+-free medium (150 mM choline) to 4.9 ± 1.3 nmoles/mg. At concentrations of 1 and 10 μM, pargyline had no effect on uptake during incubations lasting 1, 3, 5 or 10 min. Pargyline at 10^{-4} M, however, reduced uptake about 20 per cent and tranylcypramine at the same concentration prevented uptake and/or retention completely. In 1 μM pargyline, synaptosomes from rats given reserpine showed approximately half as much retention of [^{3}H]NE as controls, after incubation in 0.2 μM [^{3}H]NE for 1, 3, 5 or 10 min.

Lineweaver–Burk plots of [^{3}H]NE uptake curves are shown in Fig. 1. Variability among replicate measurements was quite small for control (placebo) samples, S.E.M. ≤ 10 per cent. The variability of reserpinized tissue was considerably greater; however, double-reciprocal values for uptake in reserpinized animals were consistently higher than placebo in the presence of lower ligand concentration. The effect of reserpine appeared to be non-competitive inhibition with little

* This study was supported by USPH Grant EYO 1655-02 and Research Career Development Award IK04 EY 00088-02 (D.A.R.).

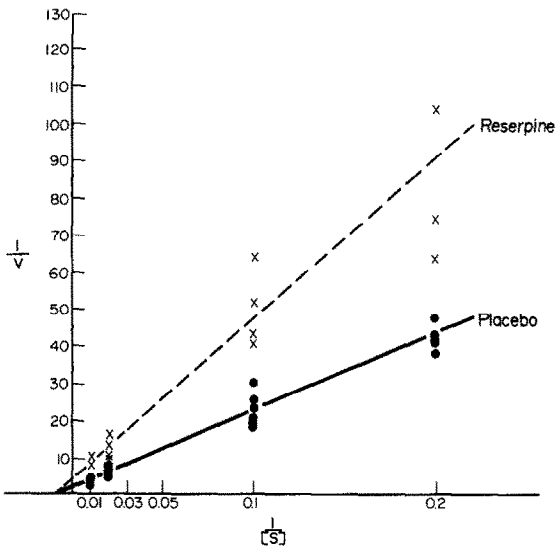

Fig. 1. Effect of reserpine *in vivo* on synaptosomal uptake of [^{3}H]NE *in vitro*. Experimental rats received 5 mg/kg of reserpine, i.p.; control rats received a volume-comparable injection of placebo. Eighteen hr post-injection synaptosomes were prepared and analyzed. The amount of uptake at 4° was subtracted from the amount of uptake observed at 37° for comparable incubation periods. $V = 10^{-9}$ moles/min/mg of protein; $[s] = 10^{-6}$ M. Each point represents the mean of three determinations.

change in K_m but a roughly 50 per cent decrease in V_{max}.

Release of [^{3}H]*NE*. For release experiments synaptosomes were generally incubated with 2 μM rather than 0.2 μM [^{3}H]NE. Filters with retained synaptosomes were transferred to a Swinnex filtering unit (Millipore Co., Bedford, MA) and perfused under positive pressure with a peristaltic pump at 12 ml/min and 37° via a 6-way valving machanism (Altex Scientific Co., Berkeley, CA) [21]. Samples of the filtrate were collected every 15 sec with a fraction collector. As shown in Fig. 2 and 3, synaptosomes were first superfused with standard (Ca^{2+}-free) buffer, containing 50 mM additional KCl or 100 μM veratridine to cause their depolarization, for 75 sec (first five samples), and then with the same solutions plus 3 mM $CaCl_2$ for a comparable period (next five samples), to permit Ca^{2+}-dependent release. Total radioactivity in the samples was determined by counting each with 20 ml of toluene–Triton X-100 phosphor.

At a concentration of 1–10 μM, pargyline in all perfusion media had no effect on the release of [^{3}H]NE from synaptosomes prepared from control animals without this inhibitor.

Figures 2 and 3 demonstrate that calcium-dependent release due to depolarization by 56 mM potassium or 100 μM veratridine was greatly diminished in synaptosomes from animals given reserpine. In potassium depolarized placebo samples, the average dis./min/collection vial increased from 8,500 to 21,000 after addition of calcium. In veratridine depolarized samples, the increase was from 7,300 to 16,000 dis./min after addition of calcium. No increase was observed in reserpinized samples. The amount of radioactivity per collection vial before and after calcium addition was 3400 and 3200, respectively, in the presence of potassium and 3500 and 3400, respectively, in the presence of veratridine. Each condition was tested in triplicate on three different experimental days (S.E.M. $\leqslant$ 10 per cent).

Release by depolarization alone, however, was approximately proportional to the synaptosomal content of [^{3}H]NE, amounting to an average of 0.9 $\pm$ 0.2 per cent/min of the content during three experiments with control synaptosomes prepared in 1 μM pargyline, and to 1.2 $\pm$ 0.2 per cent/min during three experiments with synaptosomes from reserpine-treated animals, also in pargyline.

Uptake and release of [^{14}C]*gamma-aminobutyric acid*. In three experiments, we examined the effect of reserpine on the uptake and release of gamma-aminobutyric acid (GABA), because the storage of this neurotransmitter is not known to be affected by reserpine. Samples of synaptosome were studied exactly as for [^{3}H]NE, but with 0.5 μM [^{14}C]GABA (203 mCi/m-mole; New England Nuclear Corp.) present with, or instead of, [^{3}H]NE during uptake. Amino-oxyacetic acid (0.1 mM) was also present in all media to inhibit GABA transamination [24]. The uptake of labeled GABA in synaptosomes from reserpine-treated animals averaged 102 per

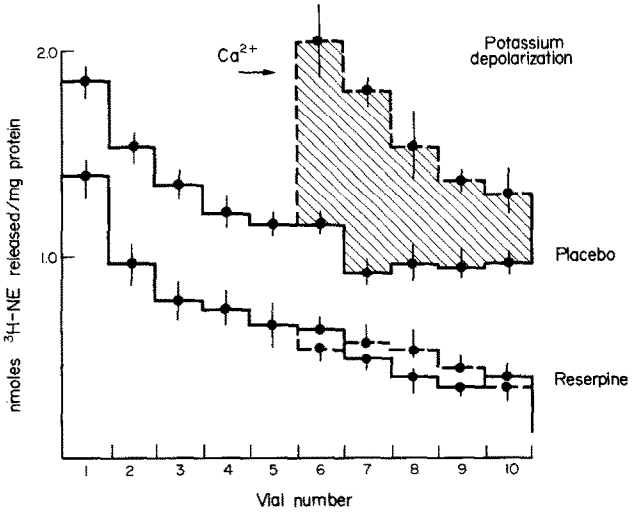

Fig. 2. Calcium-dependent, potassium-stimulated release of [^{3}H]NE from synaptosomes. Synaptosomal fractions were prepared from rat forebrain, incubated in 2 μM [^{3}H]NE and immobilized on a filter. Samples were first perfused with depolarizing levels of potassium (56.2 mM) in calcium-free medium. In half of the samples (dashed lines), calcium (3 mM) was introduced into the perfusion medium during collection periods 6–10. In the other half (solid lines), no change was made in the perfusion medium. The area between the two release curves, designated by hash marks, represents the amount of potassium-stimulated, calcium-dependent release. Data are means $\pm$ S.E.M.; n = 3.

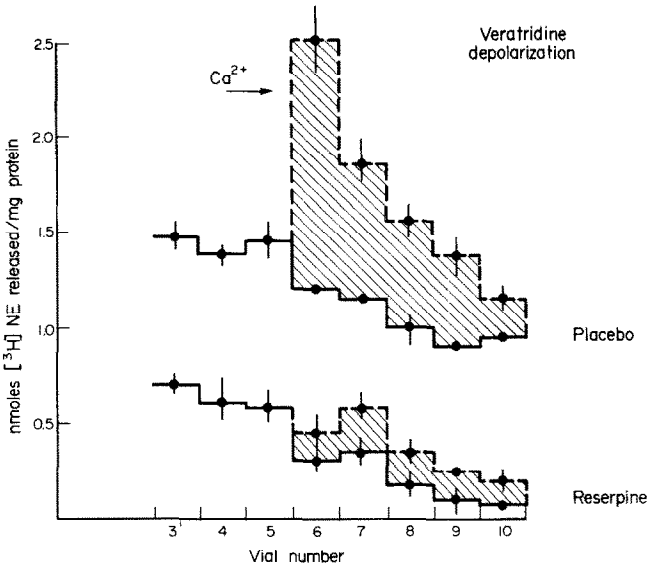

Fig. 3. Calcium-dependent, veratridine-stimulated release of [^{3}H]NE from synaptosomes. Conditions are the same as in Fig. 2, except veratridine (100 μM) was present during the perfusion as the depolarizing agent.

cent of that in controls, and release averaged 113 per cent. Release was Ca^{2+}-dependent, as expected.

We conclude, as have others before us [2, 14–17], that reserpine resistant stores of NE (protected from MAO by an inhibitor) do not readily support calcium-dependent transmitter release induced by the depolarization of nerve terminals. Certainly the effect of reserpine in inhibiting release in not due to depletion of total stores. While the exact distribution of NE between vesicles and the cytoplasm is not known for either normal or reserpine-treated terminals, it is probable that the concentration of free [^{3}H]NE achieved in the cytoplasm of our synaptosomes from reserpine-treated animals was at least as high as that in control terminals containing twice as much total amine (largely stored in vesicles). If there is any diffusion limitation for free NE within synaptosomes, then NE which has been recently taken up, as in the present experiments, should be relatively close to the surface membrane. It is unlikely, therefore, that release was diminished because of a general deficiency in cytoplasmic NE near calcium entry sites. It is known that the administration of ~1 μM reserpine *in vitro* can cause acute failure of neuromuscular transmission before there is more than 20 per cent depletion of total catecholamine stores [25, 26]. However, such blockade is fully reversible by removing free reserpine, and after 10–20 min rest, neurotransmission is normal [25] despite the presence of bound reserpine [27]. This temporary inhibition has been explained as the result of an effect of reserpine on those vesicles nearest the surface membrane. By comparison, the depletion of vesicular NE which follows the administration of reserpine to animals *in vivo* is not accompanied by major changes in neurotransmission in several tissues until the depletion of total stores exceeds 90 per cent [23, 29]. During such depletion, NE which is released from vesicles is mostly metabolized by MAO [30], although high doses can apparently raise cytoplasmic levels of NE high enough to cause some leakage to synaptic clefts, with concomitant physiological effects. The fact that dopamine β-hydroxylase is not depleted with NE [31] suggests that reserpine does not promote exocytosis. We interpret these various results as evidence that reserpine does not directly affect the normal release mechanism, a conclusion in keeping with our results concerning the release of GABA. Because the level of cytoplasmic NE also seems unimportant we favor the interpretation that vesicular NE constitutes the immediate source of transmitter for normal calcium-dependent release. Whether release is due to exocytosis or to some mechanism short of fusion of vesicles with pre-synaptic membranes remains to be established.

In summary, *in vivo* reserpine treatment blocked the evoked release of [^{3}H]NE from rat brain synaptosomes *in vitro*. Spontaneous leakage of [^{3}H]NE was increased. There was no effect on uptake or release of other neurotransmitters such as [^{14}C]GABA. These data support the hypothesis that vesicular NE constitutes the immediate source of neurotransmitter during chemical transmission.

Acknowledgements—We express our gratitude to Ms. Cheryl B. Kyles, Mrs. Diane B. Broome and R. T. Chantenez for their skilled technical assistance.

Department of Neurobiology and Anatomy, The University of Texas Medical School, Houston, TX 77025, *U.S.A.* — DIANNA A. REDBURN

Department of Psychology, University of Houston, Houston, TX 77004, *U.S.A.* — JAMES STRAMLER

Department of Pharmacology, University of Miami, School of Medicine, Miami, Fl 33152, *U.S.A.* — LINCOLN T. POTTER

REFERENCES

1. S. S. Carlson, J. A. Wagner and R. B. Kelly, *Biochemistry*, **17**, 1188 (1978).
2. L. T. Potter, *Circulation Res.* **20–21** (suppl. 3), 13 (1967).
3. L Stjarme, H. J. Schumann and G. Kroneberg, in *New Aspects of Storage and Release Mechanisms of Catecholamines*, (Eds H. J. Schumann and G. Kroneberg) p. 112. Springer, Heidelberg (1970).
4. J. E. Heuser, T. S. Reese and D. M. D. Landis, *Cold Spring Harb. Symp. Quant. Biol.* **40**, 17 (1976).
5. B. Katz, *The Release of Neural Transmitter Substances*, p. 40. Liverpool University Press, Liverpool (1969).

6. G. Burnstock and M. E. Holman, *J. Physiol, Lond.* **155**, 115 (1961).
7. A. R. Martin, *Physiol. Rev.* **46**, 51 (1966).
8. R. M. Marchbanks and M. Israël, *J. Neurochem.* **18**, 439 (1971).
9. L. A. Wagner, *Life Sci.* **17**, 1755 (1977).
10. V. S. von Euler, in *New Aspects of Storage and Release Mechanisms of Catecholamines*, (Eds H. J. Schumann and G. Kroneberg) p. 144. Springer, Heidelberg (1970).
11. L. S. van Orden, K. G. Bensch and N. J. Giarman, *J. Pharmac. exp. Ther.* **155**, 428 (1967).
12. B. Hamberger, T. Malmfors, K. A. Norberg and C. A. Sachs, *Biochem. Pharmac.* **13**, 841 (1964).
13. L. L. Iverson, J. Glowinski and J. Axelrod, *J. Pharmac. exp. Ther.* **150**, 173 (1965).
14. A. R. Wakade and S. M. Kirpekar, *J. Pharmac. exp. Ther.* **190**, 451 (1974).
15. Y. Misu and S. M. Kirpekar, *J. Pharmac. exp. Ther.* **163**, 330 (1968).
16. J. Häggendal and T. Malmfors, *Acta physiol. scand.* **75**, 33 (1969).
17. L. O. Farnebo, *Biochem. Pharac.* **20**, 2715 (1971).
18. D. G. Jones, in *Synapses and Synaptosomes*, p. 135. John Wiley, New York (1975).
19. M. P. Blaustein, E. M. Johnson and P. Needleman, *Proc natn. Acad. Sci. U.S.A.* **69**, 2237 (1972).
20. C. W. Cotman and D. A. Matthews, *Biochim. biophys. Acta* **249**, 380 (1971).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. M. S. Patterson and R. C. Greene, *Analyt. Chem.* **37**, 854 (1965).
23. J. M. Davis, F. K. Goodwin, W. E. Bunney, D. L. Murphy and R. W. Calburn, *Pharmacologist* **9**, 184 (1967).
24. D. P. Wallach, *Biochem. Pharmac.* **5**, 323 (1961).
25. V. S. von Euler, *Acta physiol. scand.* **76**, 255 (1969).
26. Y. Misu, T. Kubo and H. Nishio, *Eur. J. Pharmac.* **19**, 267 (1972).
27. S. Norn and P. A. Shore, *Biochem. Pharmac.* **20**, 2133 (1971).
28. T. E. Gaffney, C. A. Chidsey and E. Braunwald, *Circulation Res.* **12**, 264 (1963).
29. T. L. B. Spriggs, *Br. J. Pharmac. Chemother.* **26**, 271 (1966).
30. I. J. Kopin, *Handbk exp. Pharmac.* **33**, 271 (1972).
31. O. H. Viveros, L. Argueros, R. J. Connett and N. Kirshner, *Molec. Pharmac.* **5**, 69 (1969).

Biochemical Pharmacology, Vol. 28, pp. 2094–2096.
Pergamon Press Ltd. 1979. Printed in Great Britain.

Solubilization of cholinergic binding fractions from lobster axon membrane fragments

(*Received* 17 *November* 1978; *accepted* 17 *January* 1979)

During the past few years, our laboratory has been investigating the cholinergic binding properties of axonal membrane fragments from the walking leg nerves of the lobster, *Homarus americanus* [1, 2], a preparation first introduced by Denburg and O'Brien [3]. These studies have identified membrane-associated binding sites capable of interacting with [^{125}I]-α-bungarotoxin (BgTx), consistent with the requisites for a nicotinic cholinergic receptor, as well as sites capable of saturable, high-affinity binding of [^{3}H]quinuclidinyl benzilate (QNB), a highly specific muscarinic ligand. Purification and characterization of these axonal receptors require solubilization of the proteins, while retaining the native, functional form, into an environment suitable for affinity gel chromatography and other separation techniques. The present experiments were designed to survey the efficacy of a wide range of possible solubilizing conditions including salt extraction, ionic and nonionic detergents, sonication, and extraction with organic solvents [4].

MATERIALS AND METHODS

Axonal membrane fragments were prepared by the procedure of Denburg [5] and resuspended to a final protein concentration of 1–2 mg/ml in 10 mM Tris buffer, pH 7.8. The protein was incubated with a 3-fold weight excess or 1% concentration of a potential solubilizing agent for 1 hr at 5°. All studies employed initially the same criterion for solubilization, that is, protein not deposited upon centrifuging at 100,000 *g* for 60 min. Agents that solubilized a significant fraction of membrane proteins usually also caused a visually evident clearing of the microsomal suspension. It should be noted that solubilization does not exclude the possible presence of membrane micelles in the supernatant fraction.

The final supernatant fraction of solubilized proteins was dialyzed overnight against 500 ml Tris buffer at 5° to remove as much excess detergent as possible prior to determining the binding capacity of these proteins for cholinergic ligands. The binding assays utilized in this study have been described in detail elsewhere [2]. Earlier studies on the binding of cholinergic ligands to axon plasma membrane fragments yielded apparent dissociation constants (by Scatchard analysis) of 3.2×10^{-7} M for [^{3}H]nicotine [1], 1.1×10^{-7} M for [^{125}I]-α-BgTx [2], and 1.12×10^{-9} M for [^{3}H]QNB [2]. Binding to solubilized protein, therefore, was assayed at a single ligand concentration approximately twice the $K_{d_{app}}$, and binding was expressed (Table 1) in values relative to the binding capacity of untreated membrane proteins at the same ligand concentration, i.e. as per cent of nonsolubilized control.

For sonication, the membrane fragment suspension was kept at 5° in a salt–ice bath and exposed for 15–90 sec periods to sonication with a Branson probe-type sonifier.

The procedure of Sigrist *et al.* [6] was used to extract lipoproteins from the membrane fragments, with n-butanol as organic solvent in a single phase system and ether precipitation of the lipoprotein fraction. Liposomes were prepared by injecting the butanol extract into aqueous buffer solution (5 mM Tris, pH 7.8), as described by Batzri and Korn [7].

[^{3}H]Nicotine (sp. act. 250 mCi/m-mole) and [^{3}H]quinuclidinyl benzilate (sp. act. 16.4 Ci/m-mole) were purchased from Amersham Searle. [^{125}I]-α-bungarotoxin was a generous gift of Dr. Hai-Won Chang, Columbia University. Brij 36T was purchased from Emulsion Engineering, Elk Grove Village, IL. Lubrol WX was purchased several years ago from the Sigma Chemical Co. St. Louis, MO. Renex 30 was a gift of Imperial Chemical Industries, Wilmington, DE.

RESULTS AND DISCUSSION

The results of this study are summarized in Table 1. It is evident there is a wide variation of efficacy of solubilizing techniques with respect to the relative binding of nicotinic (nicotine and BgTx) and muscarinic (QNB) ligands. This supports the hypothesis of different binding sites, for these