The release of the drug from the microcapsules was related directly to the wall thickness (Fig. 6). Increasing the wall thickness retarded the release of the drug for longer than 12 hr from the unhardened microcapsules. Since the drug release rate from the hardened microcapsules is substantially lower than from the unhardened microcapsules (1), this procedure has potential for the development of a dosage form that may substantially reduce the frequency of administration, thus resulting in fewer missed doses.

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Synthesis and Preliminary Pharmacology of an Internal Standard for Assay of Neostigmine

H. E. WARD, Jr. *, J. J. FREEMAN *, J. W. SOWELL ‡, and J. W. KOSH *x

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Abstract □ The synthesis of the diethyl analog of neostigmine, its preliminary pharmacology, and its use as an internal standard for the GLC assay of neostigmine are described. Both the diethyl analog and neostigmine undergo thermal demethylation in the injection port. The column selected produced satisfactory resolution and short retention times for neostigmine and the diethyl analog. The diethyl analog apparently possesses acetylcholinesterase-inhibiting properties, as evidenced by potentiation of the contractile response to acetylcholine on the ileum. In addition, acetylcholine levels in the brain were elevated slightly. Water solutions of the diethyl analog appeared to lose biological activity with time. The diethyl analog appears to be suitable for use as an internal standard for the GLC assay of neostigmine.

Keyphrases □ Neostigmine—acetylcholine analog, synthesis and preliminary pharmacology of neostigmine analogs, quantification by GLC using flame-ionization detection □ GLC, flame ionization—analysis, neostigmine and analogs, synthesis, preliminary pharmacological studies in rats □ Cholinergics—neostigmine and analogs, synthesis, pharmacological activity evaluated in rat brain and smooth muscle, quantification by GLC

Neostigmine, a quaternary ammonium compound, has been measured following isolation from biological fluids by many methods, including chemical modification of the molecule followed by polarography (1), photocolorimetry (2), or spectrophotometry (3, 4). Other quaternary ammonium compounds have been isolated from biological fluids using ion-pair extraction (5–7). Neostigmine also has been analyzed using counterion complexation followed by liquid scintillation spectrometry (8), GLC (9, 10), or GLC–mass spectrometry (11).

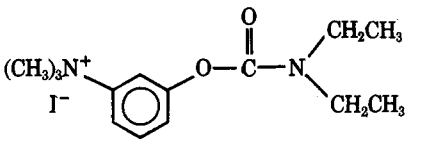
Recent assays for neostigmine used pyridostigmine as an internal standard followed by thermal dimethylation in the injection port and GLC separation. Neostigmine and pyridostigmine are both employed clinically, and their combined therapy presents a problem with currently available assays. Since GLC is widely used with substantial economic advantages over GLC–mass spectrometry, it was necessary to synthesize an analog of neostigmine applicable to the assay of quaternary ammonium cholinesterase inhibitors.

Since neostigmine possesses cholinomimetic properties, the structural similarity of the diethyl analog suggested that it may possess similar pharmacological actions. The present study reports the synthesis of the diethyl analog of neostigmine, its use as an internal standard in the quantification of neostigmine, and preliminary pharmacological findings.

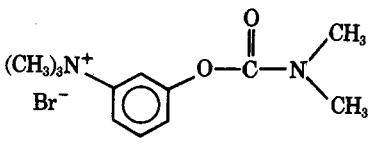
EXPERIMENTAL

Synthesis[1] of Diethyl Amino and Tertiary Analogs of Neostigmine—The preparation of the diethyl analog of neostigmine required the synthesis of an intermediate, 3-[[(diethylamino)carbonyl]oxy]-*N,N*-dimethylaniline (I), which subsequently was converted to the diethyl analog, 3-[[(diethylamino)carbonyl]oxy]-*N,N,N*-trimethylbenzenaminium iodide (II).

Synthesis of I—Compound I was synthesized by a modified procedure of Yanagisawa (12). A solution of 3-dimethylaminophenol (10.0 g, 0.073 mole) in anhydrous tetrahydrofuran (100 ml) was added dropwise with stirring to phosgene in 12.5% benzene (192 ml) in an ice bath. After 24



II



IV

[1] IR spectral data were determined on a Beckman Acculab 4 spectrophotometer using the potassium bromide technique. NMR spectra were determined on a Varian EM 360A high-resolution spectrometer with tetramethylsilane as the internal reference. Melting points were obtained using a Thomas-Hoover capillary apparatus and are uncorrected. TLC was performed using Eastman chromatogram sheets, type 6060 (silica gel); the sheets were developed in an iodine chamber. Carbon, hydrogen, nitrogen, and iodide values were obtained from analyses performed by Atlantic Microlabs, Atlanta, Ga.

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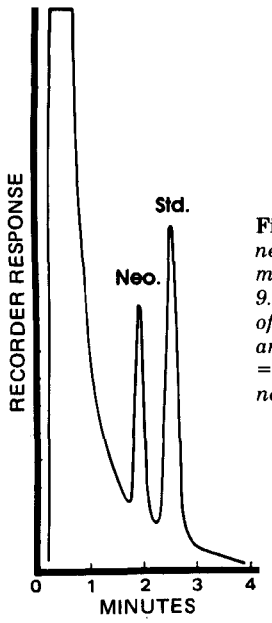

Figure 1—*Representative chromatogram of neostigmine with internal standard. Two microliters of chloroform solution containing 9.3 nmoles of neostigmine and 11.8 nmoles of internal standard was subjected to GLC analysis as described in* Experimental. *Neo = neostigmine, and Std. = diethyl analog of neostigmine (II, internal standard).*

hr at ambient temperature, the solvents and excess phosgene were removed *in vacuo*.

The residue was dissolved in tetrahydrofuran (100 ml) and was stirred in an ice bath as diethylamine (18.0 g, 0.25 mole) was added dropwise. After 12 hr at ambient temperature, the solvent and excess diethylamine were removed *in vacuo*. The residue was dissolved in chloroform (100 ml), extracted with 3 × 50-ml portions of 5% NaOH, and then extracted with several portions of saturated aqueous sodium chloride solution. The chloroform solution was dried over anhydrous sodium sulfate, and the remaining chloroform was removed *in vacuo* to yield 12.4 g of an oily residue. The IR spectrum was consistent with the assigned structure, and the oil was used for methiodide salt formation without further purification.

Synthesis of Diethyl Analog (II) of Neostigmine—A solution of I (5.0 g, 0.021 mole) in anhydrous ether (50 ml) was treated with methyl iodide (9.1 g, 0.064 mole). The methiodide salt, which precipitated over several days, was collected and dried. A portion of the crude product (4.1 g, 51.2%) was recrystallized from acetone–ethyl acetate–hexane (1:1:2) to yield a white powder. This powder was homogeneous on TLC with acetone (R_f 0.28), mp 130–131°; IR (KBr): 1715, 1420, 1270, 1230, 1210, and 1150 cm^{-1}; NMR (deuterochloroform): δ 1.25 (t, 6H, CH_3 groups of diethylamino), 3.50 (q, 4H, methylene groups of diethylamino), 3.95 [s, 9H, $(CH_3)_3N$], and 7.2–7.8 (m, 4H, aromatic H) ppm.

Anal.—Calc. for $C_{14}H_{23}IN_2O_2$: C, 44.45; H, 6.13; I, 33.55; N, 7.41. Found: C, 44.60; H, 6.14; I, 33.38; N, 7.31.

Synthesis of Tertiary Analog (III) of Neostigmine—The tertiary analog, 3-[[(dimethylamino)carbonyl]oxy]-*N*,*N*-dimethylaniline (III), was synthesized according to the procedure for I, except that dimethylamine was added instead of diethylamine.

GLC Procedures for Neostigmine (IV) and Its Analogs—Prior to chromatographic analysis, neostigmine[2] and/or its analogs were dissolved in 25 μl of chloroform. Two microliters of the chloroform solution was injected into a gas chromatograph[3] equipped with a flame-ionization detector and a 1.2-m × 2-mm i.d. glass column packed with 5% OV-17 on diatomaceous earth[4]. Assay temperatures were: column, 210°; injection port, 300°; and detector, 250°. The carrier gas was helium at a flow rate of 40 ml/min. Flow rates for air and hydrogen were 250 and 50 ml/min, respectively.

Animals and Drugs—Male albino Sprague–Dawley rats, 225–325 g, were provided with a commercial diet and water *ad libitum*.

Saline solutions of acetylcholine[2] or neostigmine were prepared immediately prior to use. Saline solutions of the diethyl analog were prepared immediately and up to 1 hr prior to use.

Measurement of Acetylcholine and Choline in Brain—Rats were implanted with intraventricular cannulas[5] (13) following anesthetization with pentobarbital (30 mg/kg) and urethan (950 mg/kg) and allowed to recover for 1–2 days. Neostigmine or the diethyl analog was administered intraventricularly in 2 μl of saline with a microliter syringe. Histological verification of cannula placement was conducted periodically using toluidine blue. Acetylcholine and choline were determined *via* GLC using ion-pair extraction followed by chemical demethylation by the method of Kosh *et al.* (14). Fifty nanomoles of butyrylcholine was added to the brain homogenate as an internal standard. Two microliters of the final chloroform extract was injected into a gas chromatograph equipped with a flame-ionization detector.

Measurement of Ileal Response—After removal of the ileal tissue, 1.25-cm segments were placed in a 15-ml tissue bath containing Tyrode's solution (15) aerated with 100% oxygen and maintained at 38°. Smooth muscle activity was monitored using a physiological recorder[6]. Following a 10-min control period, acetylcholine was added to the bath (final concentration of 2.8×10^{-6} *M*), and the response was noted. After washing with Tyrode's solution, the diethyl analog (II) was added to the bath (final concentration of 1×10^{-5} *M*). After 2 min of exposure to II, acetylcholine was introduced into the bath, and the response was compared to the effect of acetylcholine alone.

Table I—Effect of Diethyl Analog (II) and Neostigmine (IV) on Rat Brain Levels[a] of Acetylcholine and Choline

Treatment	Acetylcholine[b], nmoles/g (±*SE*)	Choline[b], nmoles/g (±*SE*)
Saline	17.2 ± 0.7	52.7 ± 1.9
II, 30 nmoles	19.3 ± 0.5*	53.5 ± 1.5
II, 60 nmoles	19.5 ± 0.8**	48.9 ± 6.6
IV, 30 nmoles	26.6 ± 0.72***	47.2 ± 5.9

[a] Male albino Sprague–Dawley rats were pretreated for 1 hr with II or IV intraventricularly in saline. [b] Brain tissue was examined for acetylcholine and choline content as described in *Experimental*. * = $p < 0.02$, ** = $p < 0.05$, and *** = $p < 0.001$; $n = 4$.

Table II—Effect of the Diethyl Analog (II) and Neostigmine (IV) on the Ileal[a] Response to Acetylcholine

Treatment	Contractile[b] Force, %	n[c]
Acetylcholine	100 ± 30.1	10
II[d] + acetylcholine	404 ± 49.5*	6
II (1-hr-old solution)	33.5 ± 84	2

[a] Ileal strips (1.25 cm) from rats were suspended in Tyrode's solution aerated with 100% oxygen and maintained at 38°. Acetylcholine and II were added to produce a final concentration of 2.8×10^{-6} and 1×10^{-5} *M*, respectively. [b] Smooth muscle contractile response to acetylcholine represents 100% (1.5 g of force). * = $p < 0.01$. [c] Number of ileal strips. [d] Compound II alone did not produce a significant response.

RESULTS

A typical GLC profile following injection of 2 μl of a chloroform solution of neostigmine (IV) (9.3 nmoles) and an internal standard [diethyl analog of neostigmine (II), 11.8 nmoles] is seen in Fig. 1. The peak forms for both IV and II were symmetrical and resolved from the solvent peak. Complete separation was obtained between the peaks for IV (retention time of 2.0 min) and II (2.7 min). Calculation of the resolution (16) of the IV and II peaks gave a value of 1.8. Column efficiency, expressed in terms of theoretical plates per foot for IV and II, was 424 and 551, respectively. The longer retention time seen for II was predicted for the OV-17 column since II contains two more methyl groups than IV (diethyl *versus* dimethyl, respectively).

The possibility of thermal demethylation of IV in the injection port was investigated by injecting the tertiary analog (III) of neostigmine. Both compounds gave identical retention times (2 min).

The effect of IV and II on acetylcholine and choline levels in rat brain is seen in Table I. Intraventricular administration of 30 nmoles of II significantly ($p < 0.02$) increased the brain concentration of acetylcholine (19.3 *versus* 17.2 nmoles/g). Increasing the dose of II to 60 nmoles also caused a significant ($p < 0.05$) but equal increase in acetylcholine concentration (19.5 ± 0.8) over that of the control. Thirty nanomoles of IV caused a more pronounced increase in acetylcholine levels than did II (26.6 ± 0.72 *versus* 19.3 ± 0.5 nmoles/g). Neither II nor IV significantly altered the choline concentration. Treatment with IV caused a noticeable

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Beckman GC-65, Fullerton, Calif.
[4] Gas Chrom Q, Applied Science Laboratories, State College, Pa.
[5] Intramedic polyethylene tubing (0.28 mm i.d.; 0.61 mm o.d.), Clay Adams, Parsippany, N.J.
[6] Physiograph DMP-4A with myograph A, Narco Bio-Systems, Houston, Tex.

anergic syndrome (ataxia, lethargy, and reduced motor activity), whereas II did not.

The effect of II on the ileal response to acetylcholine is shown in Table II. The diethyl analog alone (1×10^{-5} *M*) produced a nonsignificant increase in the frequency of spontaneous contractions compared to resting tension. The ileal response to 2.8×10^{-6} *M* acetylcholine was selected as representing 100% contraction. The contractile response to acetylcholine in the presence of II was increased more than 400% ($p < 0.01$). In addition, the frequency and amplitude of the spontaneous contractions increased compared to acetylcholine alone. Freshly prepared solutions of II appeared to be more biologically active than older ones. While freshly prepared solutions of II increased the contractile force to acetylcholine (404%), solutions prepared 1 hr prior to use decreased it (33%).

DISCUSSION

The total yield for the synthesis of II was ~60%. Most of the product was obtained over 2 days, with additional product precipitating over 2–3 weeks. The diethyl analog (II) was freely soluble in water, chloroform, methanol, and acetone and was insoluble in cyclohexanone, pentane, and hexane.

Water solutions of II appeared to turn light pink with time at room temperature, suggesting that the compound was undergoing chemical modification in solution which might alter its biological activity. This possibility was confirmed by measuring ileal contraction, blood pressure, and the elevation of brain acetylcholine by II.

In the presence of II (1×10^{-5} *M*), the contractile response to acetylcholine was increased over 400% (Table II). This response suggested that II possesses cholinesterase-inhibiting properties. However, at a higher concentration (1×10^{-3} *M*), the analog completely relaxed the ileum and abolished spontaneous rhythmicity, suggesting a more complex mechanism of action. A 1×10^{-5} *M* solution of II after 1 hr at room temperature partially blocked (33%) the effect of acetylcholine on ileal tissue.

Experiments on blood pressure demonstrated that II administered intravenously (0.025 mg/kg) possessed hypotensive activity. The vasodepressor effects of the analog were variable and diminished with the age of the solution. For instance, the hypotensive response at 5 min was −80 mm Hg, which decreased to −30 mm at 45 min. The loss of biological activity in the smooth muscle and blood pressure preparations confirms that II undergoes rapid molecular change that causes a loss of biological activity. The exact chemical or pharmacological reasons for these observations are unknown.

At both doses (30 or 60 nmoles intraventricularly), II produced an equal increase in brain acetylcholine levels (Table I). However, the elevation of acetylcholine was not as pronounced as that obtained following 30 nmoles of IV. The lower potency of II compared to IV may be due to the loss of biological activity with time since acetylcholine levels were measured at 1 hr in brain.

Compound II proved to be satisfactory as an internal standard in the GLC determination of IV (Fig. 1). The column and temperatures used provided good resolution with symmetrical peaks, which facilitated quantification. A previous report (9) indicated that IV could thermally demethylate in the injection port. Thermal demethylation under the present experimental conditions was confirmed by injecting the tertiary analog of IV, which had a retention time identical to that of IV. Furthermore, demethylation was essentially complete since chemical demethylation (14) gave equal peak heights. Although aged solutions lose their biological potency, II can be used as an internal standard for the assay of IV since GLC sensitivity is unchanged with time.

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