Effect of Sulfur Substitution for the Noncarbonyl Oxygen in Hemicholinium-3 and Acetyl-*seco*-hemicholinium-3. Synthesis, Biological Activity, and Structure-Toxicity Relationships. 2[1]

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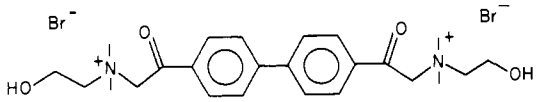
As a continuation of our efforts to develop and study inhibitors which act presynaptically on neuromuscular function, sulfur analogues of hemicholinium-3 (HC-3, 1) and acetyl-*seco*-hemicholinium-3 (AcHC-3, 3) were prepared. In each case sulfur is substituted for the noncarbonyl oxygen in HC-3 (1) and AcHC-3 (3). As expected on the basis of conformational differences between acetylcholine and acetylthiocholine both of the thio analogues are produced in the seco form and do not cyclize spontaneously or when subjected to aqueous, acidic conditions up to 100 °C. Both compounds are stable in aqueous pH 7.4 solutions at 37 °C and in slightly acidic D_2O solutions for more than 24 h. While thio-*seco*-hemicholinium-3 (11) is stable in the presence of acetylcholinesterase and butyrylcholinesterase in H_2O at pH 7.4, acetylthio-*seco*-hemicholinium-3 (12) reacts within seconds to form the *hemiacetal* form of thiohemicholinium-3 (16). Mouse toxicity studies (LD_{50}) indicate that while 12 is approximately as toxic as HC-3 (1) and AcHC-3 (3), 11 is 226 times less toxic. As in the studies with 1 and 3, mice were protected from 11 by choline and slightly by neostigmine. It is of interest, however, that almost equal and intermediate protection against 12 was afforded by choline and neostigmine. Structure-toxicity relationships of 1, 3, 11, 12, and 16 are discussed.

In 1954 Long and Schueler reported the synthesis and initial pharmacological investigations of hemicholinium-3 (HC-3, 1), a prototypical agent, which causes prejunctional inhibition in neuromuscular preparations.[2] HC-3 (1) is initially synthesized as the seco (open ring) form 2. However, on solution in water 2 rapidly undergoes intramolecular cyclization to the biologically active hemiacetal (closed ring) form 1.[2,3] In order to evaluate the



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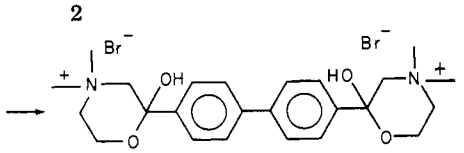

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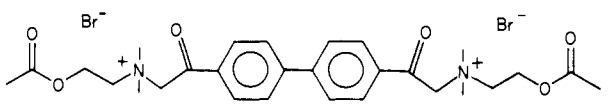
significance of cyclization on pharmacological activity, the acetate of the seco form of HC-3 (1), acetyl-*seco*-hemicholinium-3 (AcHC-3, 3) was synthesized.[4] Both 1 and



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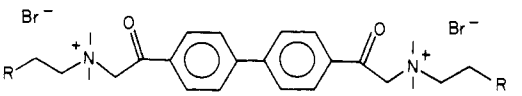
3 produce a slow depression of neuromuscular function and also inhibit cholinesterase at high concentrations.[1,5] However, unlike HC-3 (1), AcHC-3 (3) has been shown to be a potent inhibitor of choline acetylase both in vitro[6,7] and in vivo,[8] a parasympathomimetic, and an inhibitor of reuptake of neuronally released catecholamines.[4] AcHC-3 (3) slowly undergoes hydrolysis with subsequent cyclization to form HC-3 (1) in water at pH 7.4. Thus it was thought possible that a portion of the pharmacological action of 3 could derive from HC-3 (1) produced in solution. Therefore, three stable seco analogues of AcHC-3 (3)—the ether **4a**, the ketone **4b**, and the alkane **4c**—were synthesized[1] and were found to be 10–40 times less toxic than HC-3 (1) or AcHC-3 (3). Each was also found to have some HC-3-like activity, i.e., slowly developing neuromuscular blockade which could be reversed by choline. This indicated that cyclization is not necessary for this type



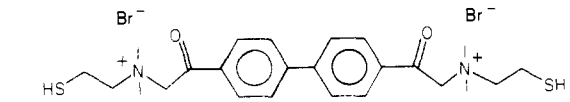
4a, R = O

b, R =

c, R =

of activity as had previously been suggested by the work of DiAugustine and Haarstad.[9] Recently an investigation of norphenyl-HC-3 (5) and a seco derivative of norphenyl-HC-3 (6) was reported which likewise indicates that cyclization is not a prerequisite to HC-3-like activity.[10] The mechanism of action of **4a–c** was also found to vary to some extent with the type of substituent. It was interesting that AcHC-3 (3) was found to bind irreversibly to acetylcholinesterase or butyrylcholinesterase without deesterification in vitro in buffered aqueous solution at pH 7.4. None of the other seco derivatives **4a–c** studied showed this characteristic and were markedly less toxic (10–40 times) than AcHC-3 (3).[1,5]

It has been shown in studies of acetylcholine (7) and several related esters that the -NCCO- grouping is in the gauche conformation both in the crystal[11] and in solution.[12] Substitution of the acyloxy oxygen of acetylcholine with either S or Se, however, leads to the trans conformation for the -NCCB- grouping in the acetylcholine derivatives **8a** and **8b** (**a**, B = S; **b**, B = Se).[13–15] Although the depolarizing abilities of **8a** and **8b** are greatly altered in various pharmacological preparations,[16–18] the molecules' roles as substrates of acetylcholinesterase are not altered.[19] Similar conformational differences exist for choline (**9**)[20] and thiocholine (**10**).[21] These conformational differences should also carry over to HC-3 (1) and AcHC-3 (3) and their acyloxy sulfur-substituted analogues. Thus, as a logical continuation of our interest in both the configurational and conformational structure-activity relationships of cholinergic compounds, an extensive investigation of thio-*seco*-hemicholinium-3 (11) and acetylthio-*seco*-hemicholinium-3 (12) annd their relationships to HC-3 (1) and AcHC-3 (3) was initiated. Herein we describe the synthesis, chemistry, and preliminary biological activity of 11 and 12 and compare them to the parent compounds 1 and 3. Structure-toxicity relationships of these compounds are also discussed. Further biological evaluation of 11 and 12 for cardiovascular activity, inhibition of



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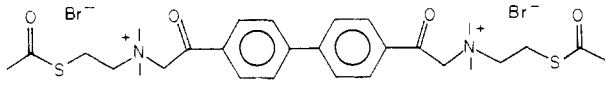

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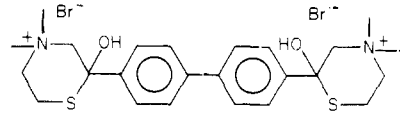
neuromuscular function, and cholinesterase and choline acetylase inhibition is in progress and will be reported subsequently.

Chemistry. Both thio-*seco*-hemicholinium-3 (**11**) and acetylthio-*seco*-hemicholinium-3 (**12**) were synthesized as the dibromide salts by the reaction of α,α'-dibromo-4,4'-biacetophenone (**13**), prepared by the method of Long and Schueler,[2] with the appropriate amines. The 2-thioacetylethyldimethylamine (**14**) required for the synthesis of **12** was obtained from 2-dimethylaminoethanethiol (**15**) by acetylation with acetic anhydride using the method of Tammelin.[22] The **15** required for this synthesis as well as the synthesis of **11** was obtained initially by the method of Hansen[23] from the reaction of ethyl sulfide with dimethylamine. The amine **15** was subsequently obtained commercially from ICN Pharmaceuticals, Inc.

The structures of **11** and **12** were confirmed by the method of synthesis, satisfactory combustion elemental analytical data, and ^{1}H NMR, ir, and uv spectral data. That **11** is, in fact, the stable, nonpolymerized thio-*seco* derivative of the oxy-hemiacetal **1** is evident from the presence in its ir spectrum of a carbonyl signal at 1674 cm^{-1} as well as a sulfur hydrogen signal at 2663 cm^{-1}. The presence of a thiol group in **11** is further confirmed by the ^{1}H NMR spectral data which, when obtained in Me_2SO-d_6, include a triplet at δ 2.08 attributable to the thio hydrogen. This signal is not observed in D_2O due to hydrogen–deuterium exchange. Further support for the presence and stability of a thiol group in **11** comes from the studies of stability in D_2O or H_2O. Interaction with DTNB confirms this. Additional support of the seco designation of **11** is derived from the uv spectrum of **11** in which the compound exhibits a λ_{max} (H_2O) of 307 nm with an ϵ max of 32 000, values which support retention of the carbonyl adjacent to the phenyl rings. It should be noted that loss of this group by formation of the hemiacetal structure shifts the λ_{max} (H_2O) to shorter wavelength, e.g., as occurs in **1** which shifts the λ_{max} (H_2O) to 262 nm.

The four compounds of interest here, HC-3 (**1**), AcHC-3 (**3**), thio-*seco*-hemicholinium-3 (**11**), and acetylthio-*seco*-hemicholinium-3 (**12**) are stable in D_2O or H_2O solutions under slightly acidic conditions or at pH 7.4 for extended periods of time. For example, slightly acidic D_2O solutions of **1** and **11** have been shown by ^{1}H NMR methods to be free of decomposition products within the limits (<0.5%) of our ^{1}H NMR spectral analysis after 20–22 h in solution at ambient temperature. Compounds **3** and **12** have been shown by ^{1}H NMR methods likewise to be free of decomposition products following 21 h at ambient temperature followed by 5.5 h at 65 °C in slightly acidic D_2O. The thio compounds **11** and **12** are both stable in aqueous pH 7.4 solutions at 37 °C for at least 48 h. Both **1** and **11** also do not react in aqueous pH 7.4 solutions in vitro in the presence of acetylcholinesterase and butyrylcholinesterase in concentrations found in blood, i.e., approximately 4 units/ml. We have previously reported[1] that AcHC-3 (**3**) undergoes rapid reaction with acetylcholinesterase and butyrylcholinesterase in H_2O at pH 7.4. This reaction is apparently an irreversible binding to the esterase without a subsequent deesterification. Under identical conditions acetylthio-*seco*-hemocholinium-3 (**12**) also undergoes a rapid interaction with acetylcholinesterase and butyrylcholinesterase. This interaction, however, does not appear to be irreversible. Instead, **12** appears to undergo immediate deesterification followed by cyclization to the hemiacetal form of thiohemicholinium-3 (**16**). This proposed mechanism is supported in two major ways.



16

First, when 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (**17**) is added to an aqueous solution of **12**, no reaction occurs. Conversely, when DTNB (**17**) is added to an identical solution of **11** a reaction between DTNB (**17**) and the nonesterified, nonpolymerized thiol group of **11** begins immediately at a rapid rate. If either esterase is added to the solution of **11**, the rate of reaction of **11** with DTNB (**17**) is not altered. However, if the esterase is added to the solution of **12** in which no reaction was previously noted, reaction with DTNB (**17**) begins immediately at a rate comparable to that observed for **11** and DTNB (**17**) indicating that deesterification of **12** to produce an easily accessible sulfur atom has occurred. Secondly, upon addition of esterase to a solution of **12** there is a large hypsochromic shift observed in the uv spectrum of **12** from a λ_{max} (H_2O, pH 7.4) of 310 nm to a λ_{max} (H_2O, pH 7.4) of 264 nm. It should be noted that a hemiacetal form of HC-3 (**1**) has a λ_{max} (H_2O, pH 7.4) of 262 nm. This indicates that the carbonyl groups conjugated to the phenyl rings are no longer present and that in fact the thiohemicholinium-3 (**16**) thus produced is in the hemiacetal form. The implications of this will be discussed in the pharmacological and structure–toxicity relationship section of this paper.

As discussed previously the -NCCO- grouping of acetylcholine (**7**) has been found to be in the gauche conformation both in the crystal[11] and in solution[12] while the -NCCS- grouping of acetylthiocholine (**8a**) is in the trans conformation.[13-15] One finds similar conformational differences for choline (**9**)[20] and thiocholine (**10**).[21] These conformational differences should carry over to HC-3 (**1**) and AcHC-3 (**3**) and their acyloxy sulfur-substituted analogues **11** and **12**. This appears to be the case in the thio-HC-3 (**11**) that was synthesized in the seco form and which resisted all attempts at cyclization. The acetylthiohemicholinium-3 (**12**) likewise is synthesized in the seco form and, due to the presence of the acetyl substituent, does not cyclize. It is of interest that in vitro enzymatic deesterification of **12** does yield the hemiacetal form of thiohemicholinium-3 (**16**). It is unfortunate, however, that this means of preparing thio-HC-3 (**16**) for obvious reasons cannot be employed to prepare sufficient quantities of **16** for direct biological evaluation.

Pharmacology and Initial Structure–Toxicity Relationships. Table I summarizes LD_{50} studies in Charles River CD-1 adult male albino mice (20–30 g). Doses of the respective compounds in normal saline (0.9% NaCl) were injected ip. The animals which subsequently died began to show effects within 5 min or less. These effects included exophthalmos, mild to moderate ataxia, respiratory difficulties, loss of righting response, SLUD[24] syndrome, and clonic convulsions. Immediate autopsy revealed that cardiac contractions had not ceased and that peristalsis of the small intestines had increased. Examination of the lungs and peritoneal cavity revealed no signs

Table I. Mouse Toxicity Studies

Compd	LD_{50}, mg/kg ip	95% confidence limits	Ratio of LD_{50} doses to that of HC-3
HC-3 (1)	0.13[a]	0.11-0.17[a]	1.0
AcHC-3 (3)	0.125[a]	*a*	1.0
Thio-AcHC-3 (12)	0.133	0.12-0.14	1.0
Thio-*seco*-HC-3 (11)	29.4	28.6-30.2	226

[a] See ref 4.

Table II. Antagonism by Choline and Neostigmine of Drug Toxicity in Mice

Compd	LD_{95} of compd, mg/kg ip	Antagonist[a]		% mortality
		Choline, mg/kg ip	Neostigmine, mg/kg ip	
HC-3 (1)	0.2	20		15
			0.2	90
AcHC-3 (3)	0.15	20		0
			0.2	80
Thio-AcHC-3 (12)	0.16	20		40
			0.2	30
Thio-*seco*-HC-3 (11)	34.5	20		10
			0.2	70

[a] Antagonist was administered 1 min prior to compound.

of excess fluids. Thus, the apparent cause of death was respiratory failure.

Both HC-3 (1) and AcHC-3 (3) have an LD_{50} of 0.13 mg/kg.[4] The other two compounds of interest, i.e., thio-AcHC-3 (12) and thio-*seco*-HC-3 (11), were equally toxic and 226 times less toxic, respectively. We have noted previously that the basis of the toxicity of 1 and 3 is not the same.[1] The toxicity of 1 appears to be directly related to the cyclic structure of the nonaromatic moieties of the molecule. The toxicity of 3 is best explained on the basis of the ability of a form of 3, quite likely an acetylcholinesterase–AcHC-3 complex, to inhibit choline acetyltransferase.[1] The similar toxicity of 12 is explained by the in vitro experiments which show that 12 undergoes enzymatic deesterification with concomitant cyclization to form thio-HC-3 (16), the thio analogue of the hemiacetal HC-3 (1). The same reaction would be expected in vivo and the 16 thus formed would be expected to have pharmacological properties quite similar to those of 1 and thus similar toxicity. It is interesting that the seco form of thio-HC-3 (11) is 226 times less toxic than 1, 3, and 12. Choline acetyltransferase inhibitory studies are currently being conducted with 11 and 12. Preliminary results indicate that thio-*seco*-HC-3 (11) has significantly less choline acetyltransferase inhibitory activity than 1, 3, or 12. Although the mechanism of lethality of 11 is still not certain, the decreased lethality of 11 relative to 1, 3, and 12 appears to be a result of the molecule's inability to cyclize into a thio-hemiacetal structure associated with a reduction of choline acetyltransferase inhibitory activity. Thus it appears that, at least in the case of hemicholinium-like functional isomers such as 11 and 16, cyclization greatly enhances toxicity.

Studies of antagonism by choline and neostigmine of drug toxicity in Charles River CD-1 adult male albino mice (20–30 g) were conducted and are summarized in Table II. In each case 20 mg/kg of choline or 0.2 mg/kg of neostigmine (doses which are nonlethal in control animals) was administered to the animal 1 min prior to administration of an ip (>LD_{95}) dose of the HC-3 (1) analogue. As in the studies with HC-3 (1) and AcHC-3 (3), mice were protected from thio-*seco*-HC-3 (11) by choline. Also, there was a slight amount of protection afforded by neostigmine. It is of interest, however, that an almost equal, though intermediate, degree of protection against the thio-AcHC-3 (12) was afforded by choline and neostigmine. Thus, substitution of a sulfur atom in the molecule in place of the ether oxygen appears to alter the mechanism of action of hemicholinium analogues.

Experimental Section

All melting and decomposition points were determined on a Fisher-Jones hot-stage melting point apparatus and are uncorrected. Boiling points were observed during distillation and are likewise uncorrected. Infrared spectra were recorded in Beckman IR-10 and Perkin-Elmer 337 and 257 spectrophotometers and were calibrated against polystyrene. The ultraviolet spectra and extinction coefficients were obtained on a Beckman DB spectrophotometer. Proton magnetic resonance spectra were determined on a Varian A-60 spectrometer using D_2O as the solvent with 1% tetramethylsilane as an external standard unless otherwise indicated. All refractive indices were observed on a Bausch and Lomb refractometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., are indicated as empirical formulas, and are within ±0.4% of the theoretical values.

α,α′-Dibromo-4,4′-biacetophenone (13). Bis(phenacyl bromide) 13 was prepared by the method of Long and Schueler:[2] mp 223.9–224.4 °C.

2-Dimethylaminoethanethiol (15). The amine 15 was prepared according to the procedure of Hansen:[23] bp 57–58 °C (61–64 mmHg) [lit. 58–59 °C (63 mmHg)]; $n^{25}D$ 1.4640 (lit. $n^{25}D$ 1.4630).

2-Thioacetylethyldimethylamine (14). The method of Tammelin was utilized to prepare 14:[22] bp 83–84 °C (22 mmHg) [lit. 78 °C (14 mmHg)]; $n^{25}D$ 1.4747 (lit. $n^{25}D$ 1.4763).

4,4′-Biphenylylenebis(2-oxoethylene)bis(2-thioacetylethyldimethylammonium bromide) (12). Bis(phenacyl bromide) 13 (1.497 g, 0.0038 mol) was dissolved with stirring at ambient temperatures in a minimum of THF. To this was added a THF solution of 2-thioacetylethyldimethylamine (14) (1.328 g, 0.0098 mol). After stirring the solution overnight in a sealed flask the resulting precipitate was collected, washed with THF followed by Et_2O, and recrystallized from absolute MeOH to give 0.948 g (37%) of 12: mp 222–225 °C dec; ir (KBr) ν 1690 (carbonyl), 1604 (phenyl), 1402 (carbonyl methylene), 822 (*p*-phenyl), 625 (sulfur carbon), and 592 cm^{-1} (amine); uv λ_{max} (H_2O) 310 nm (ε 36000); ^1H NMR (D_2O) δ 7.96–7.81 (8 H, aromatic), 3.40 (s, 12 H, nitrogen methyl), 4.20–3.20 (multiple overlapping signals, 12 H, methylenes adjacent to nitrogen and carbonyl and one carbon removed from nitrogen), 2.31 (s, 6 H, methyl of thioacetyl). Anal. ($C_{28}H_{38}Br_2N_2O_4S_2$) C, H, N.

4,4′-Biphenylylenebis(2-oxoethylene)bis(2-thioethyldimethylammonium bromide) (11). A total of 2.0 g (0.0051 mol)

of bis(phenacyl bromide) 13 was dissolved in a minimum of THF with stirring at ambient temperature. To this was added neat 2.0 g (0.020 mol) of 2-dimethylaminoethanethiol (15). Stirring was continued overnight in a sealed flask and the resulting precipitate was isolated, washed with THF followed by Et_2O, and recrystallized from absolute MeOH to yield 2.4 g (78%) of 11: mp 184.2–184.8 °C; ir (KBr) ν 1674 (carbonyl), 1602 (phenyl), 1399 (carbonylmethylene), 812 (*p*-phenyl), 637 (sulfur carbon), 564 cm^{-1} (amine); uv λ_{max} (H_2O) 307 nm (ϵ 32000); ^1H NMR (D_2O) δ 7.68–6.99 (8 H, aromatic), 3.40–2.74 (overlapping signals, 12 H, methylenes adjacent to nitrogen and carbonyl and one carbon removed from nitrogen), 2.87 (s, 12 H nitrogen methyl); ^1H NMR (Me_2SO-d_6) δ 2.08 (t, 2 H, thiol). Anal. ($C_{24}H_{34}Br_2N_2O_2S_2$) C, H, N, S.

Stability of Thio-*seco*-hemicholinium-3 (11) and Acetylthio-*seco*-hemicholinium-3 (12) in D_2O. Procedure A. A deuterium oxide solution of 11 (0.05 M) was prepared. The solution as well as a solvent blank was immediately analyzed utilizing a 60-MHz ^1H NMR spectrometer. The solution and blank were allowed to stand for 20–22 h at ambient temperature after which time each was again analyzed. The solution was found to be free of decomposition products within the limits (<0.5%) of our ^1H NMR spectral analysis.

Procedure B. A similar solution of the ester 12, which would be expected to be the most water labile analogue, was prepared and initially analyzed as in procedure A. The solution was then maintained at ambient temperature and ^1H NMR analysis was conducted at intermittent times up to 21 h. The solution was then heated to 65 °C for 5.5 h and analyzed by ^1H NMR which indicated that within the limits previously described the solution was free of decomposition products.

Stability of Thio-*seco*-hemicholinium-3 (11) and Acetylthio-*seco*-hemicholinium-3 (12) in H_2O at pH 7.4. Aqueous solutions of 11 and 12 (1.00×10^{-5} M) buffered to pH 7.4 were prepared. The uv absorption spectrum of each was immediately recorded utilizing an identically buffered aqueous sample as a reference. Each sample was maintained at 37 °C for 48 h. The uv spectrum of each was subsequently recorded at various intervals during this time. No change was observed in the absorption spectrum of 11 or 12.

Interaction of Thio-*seco*-hemicholinium-3 (11) and Acetylthio-*seco*-hemicholinium-3 (12) in H_2O at pH 7.4 with Acetylcholinesterase and Butyrylcholinesterase. Procedure A. Buffered (pH 7.4) aqueous solutions of 11 and 12 (1.00×10^{-5} M) were prepared. Employing an identically buffered aqueous sample as a reference, the uv absorption spectrum of a 1-ml aliquot of each was immediately recorded. A 0.1-ml aliquot of 40 units/ml buffered (pH 7.4) aqueous solution of acetylcholinesterase was then added to the sample and reference to produce an enzymic concentration similar to that found in blood. The uv absorption spectrum was immediately recorded. An examination of the spectra of thio-*seco*-hemicholinium-3 (11) recorded before and after addition of enzyme showed no shift in the λ_{max} (H_2O, pH 7.4) of this compound following enzymic addition. An examination of equivalent spectra for acetylthio-*seco*-hemicholinium-3 (12) revealed a hypsochromic shift of the λ_{max} (H_2O, pH 7.4) from 310 to 264 nm. Thus no reaction of 11 with the enzyme was observed. Although reaction of 12 with the enzyme did occur, the expected seco form of 11 was not produced. Instead, the hemiacetal form 13 was observed. Each solution was then incubated at 37 °C for 6 h and the uv absorption spectrum was again recorded. No significant changes occurred in the spectra after 6 h.

Procedure B. The experiment described in procedure A above was repeated substituting butyrylcholinesterase for acetylcholinesterase. Equivalent results were obtained.

Attempted Cyclization of Thio-*seco*-hemicholinium-3 (11). An aqueous solution of thio-*seco*-HC-3 (11) (1.0×10^{-5} M) was prepared and the uv spectrum was obtained. To 1 ml of this solution was added 1 mequiv of HCl. The sample was shaken and analyzed by uv, heated to 50 °C for 10 min, and again analyzed and heated to 100 °C for 5 min and reanalyzed. In each case no change was observed in the spectrum, indicating that cyclization had failed to occur.

Interaction of Thio-*seco*-hemicholinium-3 (11) and Acetylthio-*seco*-hemicholinium-3 (12) in H_2O at pH 7.4 with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) (17). Procedure A. Aqueous buffered (pH 7.4) solutions of 11 and 12 (1.0×10^{-3} M) were prepared. To each was added 100 μl of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 17). The absorbance at 412 nm was recorded employing an identically buffered aqueous sample containing DTNB (17) as a reference. Upon addition of DTNB (17) to the sample containing 11 an immediate reaction is observed between 11 and DTNB (17) indicating the presence of a free thiol group in 11. Conversely no reaction is observed between the thioester group of 12 and DTNB (17).

Procedure B. Solutions identical with those utilized in procedure A were prepared. A total of 100 μl of a solution 0.01 M in DTNB and containing 0.025 unit of acetylcholinesterase was added to an identically buffered aqueous sample for use as a reference and to each of the samples containing 11 or 12. The absorbance of each sample was recorded at 412 nm. No change in the rate of reaction of 11 with DTNB (17) from that found in procedure A was observed. In the case of 12, in contrast to what was observed in procedure A, reaction with DTNB (17) begins immediately in procedure B and proceeds at a rate comparable to that observed for 11, indicating that deesterification of 12 to produce an easily accessible sulfur atom has occurred.

Procedure C. The experiment described in procedure B above was repeated substituting butyrylcholinesterase for acetylcholinesterase. Equivalent results were obtained.

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