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THE ACTIVE STRUCTURE OF HEMICHOLINIUM INHIBITING THE BIOSYNTHESIS OF ACETYLCHOLINE*†

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Abstract—Four methyl analogs of hemicholinium (HC-3) were shown to undergo hemiacetalization, as was previously shown for the parent compound. These analogs produced hemicholinium-like responses with relative activities either equal to or lower than those of HC-3. Hemiacetalization was found to be restrained in one analog, which consisted of a mixture of opened-ring (seco) and closed-ring (hemiacetal) tautomers (desmotropes). The preparative separation of the seco-hemiacetal desmotropes was accomplished by counter current distribution of column chromatography. The isolated seco desmotrope had a λ_{max} at 292 mμ (log ϵ 4·49) in the ultraviolet spectrum; the isolated hemiacetal desmotrope had a λ_{max} at 257 mμ (log ϵ 4·42) equal to that of HC-3. Findings of paper and thin-layer chromatograms were in agreement with the presence of two components of the unseparated desmotropic mixture. The lack of spontaneous ring closure of the seco material in aqueous solutions to form the hemiacetal, and *vice versa*, made possible a comparison of the pharmacology of these compounds. Only the hemiacetal desmotrope qualified in all evaluations as a hemicholinium type of compound. The results maintain the thesis that for HC-3 *per se* the hemiacetal structure is requisite for inhibition of acetylcholine biosynthesis and that any restraint on hemiacetalization will lead to a reduction in activity.

HEMICHOLINIUM (HC-3) has been shown to interfere with cholinergic transmission in the peripheral nervous system. In low doses and under conditions that place a high demand upon the source of transmitter, HC-3 appears to attack only the presynaptic processes of ganglia[1] and neuro-muscular junctions.[2–4] In the case of autonomic ganglia, HC-3 greatly reduced the acetylcholine (ACh) content as well as the ACh liberated from presynaptic terminals.[1, 5–8] Choline has been found to be a specific and dramatic antagonist to HC-3-induced poisoning[9, 10] and neuromuscular blockade.[11, 12] MacIntosh *et al.*[1] and Gardiner[13] have reported that HC-3 is a potent inhibitor of ACh biosynthesis by minced brain and acts at some stage before the acetylation of choline by choline acetylase (acetyl-CoA: choline *O*-acetyltransferase). They also postulated that HC-3 blocked an active concentrating mechanism for choline by nerve cells and thereby slowed ACh synthesis. Recently, Hodgkin and Martin[14] and

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Schuberth *et al.*[15] gave support to this mechanism by showing that HC-3 inhibits the active uptake of isotopically labeled choline into nerve tissue *in vitro*.

This unique pharmacological character has spurred an interest in HC-3 on a structure–activity basis. HC-3 was originally reported by Long and Schueler[16] as the chain type of structure shown in Fig. 1 (structure A). Subsequently work by Schueler[9] established that the compound underwent keto-hemiacetal (ring-chain) tautomerism, forming the cyclic hemiacetal shown in Fig. 1 (structure B). Some or all of the four

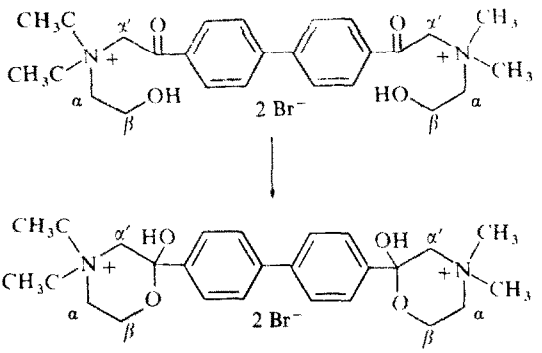


FIG. 1. The conversion of seco-HC-3 (A) to hemiacetal-HC-3 (B).

choline-like moieties of HC-3 which arise through hemiacetalization might allow HC-3 to bind readily to a choline transport system at nerve terminals. Pharmacological studies so far have indicated that the biphenyl nucleus of HC-3 can be modified without serious loss of hemicholinium-like activity.[17–19] The bisected HC-3 molecule, which is exactly half of structure B shown in Fig. 1, was found by Schueler[9] to be essentially a nontoxic congener. Relatively minor structural changes of the cationic heads of HC-3 either reduced markedly or abolished hemicholinium-like activity.[9, 16, 17, 20]

One purpose of this investigation was to study the participation of the choline moieties of HC-3 in biological systems by selective introduction of methyl groups into those moieties. As will be shown, methyl substitutions on the morpholinium rings of HC-3 produced analogs having marked differences in activity.

During the course of this investigation another purpose unfolded. Prior to recrystallization, an analog of HC-3 unexpectedly yielded an isolatable opened-ring tautomer. This opened-ring tautomer, unlike that of HC-3, was stable in aqueous solutions and therefore could be compared pharmacologically with its corresponding closed-ring tautomer. The results of this comparison along with other related observations led to a proposal of the active structure of HC-3 that inhibits the biosynthesis of ACh.

METHODS

Melting points

All melting points were taken on a hot stage (Fisher–Johns) melting points apparatus and are uncorrected.

Ultraviolet spectra

Ultraviolet spectra were obtained on a Beckman DB-G grating spectrophotometer

with a Beckman 10-in. recorder. Except where indicated otherwise, compounds were dissolved in 95% ethanol.

Infrared spectra

Infrared spectra were recorded on a Beckman Microspec spectrophotometer. Compounds examined were mixed appropriately with anhydrous KBr in a Wig-L-Bug dental amalgamator and then pelleted in a Mini-Press (Wilks Scientific Company).

Nuclear magnetic resonance spectra

The n.m.r. spectra were taken with a Varian spectrometer, model A-60.

Microanalyses

Microanalyses were carried out by Alfred Bernhardt (Muhlheim, Germany) and Galbraith Laboratories, Inc. (Knoxville, Tenn.). Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within $\pm$ 0·4 per cent of the theoretical values.

Refractive indices

All aminoalcohols used in the synthesis of compounds I, II, III and IV were examined routinely for refractive index with an Abbe refractometer (Bausch & Lomb) at the temperatures indicated.

Molecular weight determinations

Molecular weight determinations were carried out by Galbraith Laboratories, Inc. (Knoxville, Tenn.). An osmometric method was used, which measured the temperature difference caused by condensation of solvent vapors into a drop of solvent and a drop of solution of the compound held in a constant temperature chamber saturated with solvent vapors. Ethanol was the solvent used in this study.

Paper chromatography

One-dimensional ascending paper chromatography was carried out on Whatman no. 20 paper with *n*-butanol–ethylene glycol–water (4:1:1) as a solvent system. Samples to be chromatographed were dissolved in methanol. Development was allowed to proceed until the solvent front was 14 cm from the origin. The spots were made visible by spraying with potassium iodoplatinate reagent.[21]

Thin-layer chromatography

A solvent system of *n*-butanol–glacial acetic acid–water (10:1:1) was used on silica gel thin-layer glass fiber sheet (Gelman Company, type SG). For spot detection, the chromatogram was air-dried in a hood; the sheet was sprayed with concentrated sulfuric acid and placed on a hot plate.[22] Organic materials appeared on the sheet as charred, dark-brown spots.

Column chromatography

A cellulose chromatographic column was prepared by mixing 200 g of fibrous cellulose powder (Whatman Column Chromedia CF 11) with enough of the *n*-butanol–ethylene glycol–water (4:1:1) or chloroform–methanol (10:1) eluent to make a homogenous slurry. The slurry was then poured slowly into a Chromaflex column support

and allowed to pack. The resulting dimensions of the column beds were 41 ± 1 by 4 cm. The appropriate fractions were evaporated to dryness under reduced pressure in a rotary evaporator.

Synthesis of the compounds

α,α′-Dibromo-4, 4′-biacetophenone. The method of Long and Schueler[16] was followed. However, tetrahydrofuran was used to recrystallize the product giving a 72 per cent yield, m.p. 225–227° (lit.[16] m.p. 226–227°).

α,α′-Dibromo-4, 4′-bipropionylphenone. The above method for the preparation of α,α′-dibromo-4, 4′-biacetophenone was used, with the exception that an equimolar amount of 2-bromopropionyl bromide replaced bromoacetyl bromide giving a 76 per cent yield, m.p. 176–177° (lit.[23] m.p. 174–179°).

2, 2′-(4, 4′-*Biphenylylene*) *bis*(2-*hydroxy*-4, 4-*dimethylmorpholinium bromide*) (*HC*-3). The method of Long and Schueler[16] (tetrahydrofuran was used as the reaction solvent) was followed. Recrystallization from ethanol–ether gave the product as a white solid. The yield and properties of this compound are summarized in Table 1.

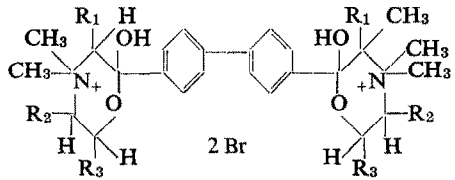
2, 2′-(4, 4′-*Biphenylylene*)*bis*(2-*hydroxy*-3, 4, 4-*trimethylmorpholinium bromide*) (*I*). 2-Dimethylaminoethanol was purified by double distillation [b.p. 133–135° n_D^{20} 1·4290(lit.[24] b.p. 135° n_D^{20} 1·4300)] and then 3·6 g (0·048 mole) was added to a stirred solution of 1·5 g (0·0038 mole) of α,α′-dibromo-4, 4′-bipropionylphenone dissolved in 100 ml of hot tetrahydrofuran (THF). The mixture was refluxed for 30 min and then cooled to room temperature. The solid which formed was collected on a filter, washed with THF and then ether, and dried at 50° *in vacuo*. Recrystallization from ethanol–ether gave the product as white solid. The yield and properties of I are summarized in Table 1.

2, 2′(4, 4′-*Biphenylylene*) *bis* (2-*hydroxy*-4, 4, 5-*trimethylmorpholinium bromide*) (*II*). 2-Dimethylamino-1-propanol was purified by double distillation (b.p. 146–180° n_D^{27} 1·4349) and then 5 g (0·048 mole) was added to a stirred solution of 1·5 g (0·0038 mole) of α,α′-dibromo-4, 4′-biacetophenone dissolved in 100 ml of hot THF. The mixture was refluxed for 30 min and then cooled to room temperature. The solid which formed was collected on a filter, washed with THF and then ether, and dried at 50° *in vacuo*. Recrystallization from ethanol–ether gave the product as a white solid. The yield and properties of II are summarized in Table 1.

2, 2′-(4, 4′-*Biphenylylene*) *bis* (2-*hydroxy*-4, 4, 6-*trimethylmorpholinium bromide*) (*III*). The method for the preparation of II was used, with the exception that 2-dimethylamino-1-propanol was replaced with an equimolar amount of 1-dimethylamino-2-propanol [b.p. 125–126° n_D^{23} 1·4190(lit.[25] b.p. 124–126° n_D^{23} 1·4218)]. The yield and properties of III are summarized in Table 1.

2, 2′-(4, 4′-*Biphenylylene*)*bis* (2-*hydroxy*-4, 4, 5, 5-*tetramethylmorpholinium bromide*) (*IV*). The method for the preparation of II was used, with the exception that 2-dimethylamino-1-propanol was replaced with an equimolar amount of 2-dimethylamino-2-methyl-1-propanol b.p. [159–161° n_D^{27} 1·4442(lit.[25] b.p. 160–161° n_D^{27} 1·4442)]. The yield and properties of IV are summarized in Table 2. The chemical name for IV as it appears above refers to that product (hemiacetal-IV) of the reaction which underwent complete hemiacetalization. Prior to recrystallization, about 40 per cent of the total product, as determined by countercurrent distribution measured with ultraviolet analyses, remained uncyclized as [4, 4′-biphenylylenebis(2-oxoethylene)]

TABLE 1. METHYL ANALOGUES OF HEMICHOLINIUM NO. 3



Compound	R_1	R_2	R_3	M.p. (C°)	Yield (%)	Formula*†	R_f†‡	LD_{50}‡§ (mg/kg)	I_{50}AChE, (M)
HC-3	H	H	H	226–228, dec*§	79	$C_{24}H_{34}Br_2N_2O_4$	0·44	0·063 (0·048–0·082)	$>10^{-3}$(37% at 1×10^{-3})
I	CH_3	H	H	185–188, dec	71	$C_{26}H_{38}Br_2N_2O_4$	0·55	0·065 (0·050–0·083)	$>10^{-3}$(8% at 1×10^{-3})
II	H	CH_3	H	190–192, dec	80	$C_{26}H_{38}Br_2N_2O_4$	0·45	0·145 (0·120–0·173)	$6{\cdot}0 \times 10^{-4}$
III	H	H	CH_3	185–188, dec	68	$C_{26}H_{38}Br_2N_2O_4$	0·77	0·52 (0·42–0·64)	$5{\cdot}6 \times 10^{-4}$

* All compounds were analyzed for % C, H. HC-3 and compounds I, II and III analyzed as monohydrates even after high-vacuum drying at 100°.
† Solvent system was *n*-butanol–ethylene glycol–water (4:1:1) on Whatman no. 20.
‡ The numbers in parentheses represent the 95 per cent confidence limits.
§ HC-3 has been found by V. B. Haarstad and F. W. Schueler (unpublished data) to crystallize in variable hydration states with different decomposition points. This water of crystallization is very difficult to remove from HC-3. These properties probably also occur for compounds I–III.

TABLE 2. SOME PROPERTIES OF SECO- AND HEMIACETAL-IV

Compound*	M.p. (C°)	95% Ethanol λ_{max} (mμ)	Infrared carbonyl absorption (5·9μ)	R_f in solvent systems† S_1	S_2	LD_{50}† (mg/kg)	I_{50} AChE (M)
IV§ (desmotropic mixture)	183–185, dec	260–290‖	strong	0·62 and 0·86	0·75 and 0·88	0·115 (0·092–0·142)	$9·3 \times 10^{-5}$
Seco-IV ‡‡,**	182–185, dec	292	very strong	0·86	0·88	10·1 (7·7–13·2)	$2·7 \times 10^{-4}$
Hemiacetal-IV ‡‡,††	182–184, dec	257	nil	0·62	0·75	0·058 (0·047–0·069)	$5·1 \times 10^{-4}$

* Formula (theoretical for this series is $C_{28}H_{42}Br_2N_2O_4$. All compounds were analyzed for % C, H.

† Solvent systems were as follows: S_1, *n*-butanol–ethylene glycol–water (4:1:1); S_2, *n*-butanol–glacial acetic acid–water (10:1:1) on silica gel thin-layer glass fiber sheet (Gelman Company, type SG).

‡ The numbers in parentheses represent the 95% confidence limits.

§ Analyzed within ± 0·4% of the calcd. % C and H without recrystallization. All values obtained were with a sample containing approximately 40 % seco- and 60% hemiacetal-IV as determined by countercurrent distribution measured with ultraviolet analyses.

‖ Broad single- or double-peaked absorption, depending on the duration of reflux time of the reaction (see Fig. 4.)

** Analysis C: calcd, 53·34%; found, 55·17 %. Analysis H: calcd, 6·71 %; found, 5·76%. Molecular weight: calcd, 630; found, 599.

†† Isolated from the desmotropic mixture by countercurrent distribution.

‡‡ Analyzed as the hemihydrate.

bis[(2-hydroxy-1, 1-dimethylethyl) dimethylammonium bromide] (seco-IV). The yield and properties of the desmotropic mixture IV (seco-hemiacetal-IV) are summarized in Table 2.

When the reaction was carried out at room temperature for 24 hr, the amount of seco-IV increased to about 60 per cent of the total product mixture. Recrystallization of the desmotropic mixture from ethanol–ether greatly reduced the proportion of seco-IV and was avoided in order to improve the yield of this desmotrope. With care, the mixture could be prepared in an analytically pure form without resorting to recrystallization.

Separation and isolation of seco- and hemiacetal-IV

A countercurrent distribution[26] was used with thirteen tubes (12-plate transfer) each containing preequilibrated *n*-butanol (20-ml) and distilled H_2O (20 ml). The bottom layer (mobile phase) was moved manually with a 20-ml syringe equipped with a 5 in. blunted trocar. One g of the mixture of desmotropes was used for countercurrent separation. Because of the surface-active properties of seco-IV, centrifugation (1500–2000 rpm for 30 min) was necessary to restore the butanol–H_2O interface after shaking the tubes. After the last transfer step, the content of each tube was made into a single phase by the addition of 10 ml of 95% ethanol. Aliquots were taken from each tube and diluted with H_2O for ultraviolet spectroscopy, with H_2O as an optical reference. Tables of binomial probability distribution were used to compute theoretical countercurrent values. Tubes 3 and 4, and 11 and 12 were used routinely to isolate seco- and hemiacetal-IV respectively. The tube contents were taken to dryness under reduced pressure in a rotary evaporator at 50°. The residue was recrystallized from ethanol–ether, filtered, washed (THF–ether) and dried *in vacuo* at 50°. Both desmotropes were obtained as white solids. The properties of the isolated desmotropes are summarized in Table 2.

Pharmacology

Toxicity studies. The 24-hr LD_{50} values were determined in white female mice (Charles River) weighing 20–25 g. The compounds were dissolved in saline and injected intraperitoneally (i.p.) in a volume equivalent to 10 ml/kg body weight. Three groups of mice, consisting of ten mice per group, were used for each determination. The LD_{50} and its 95 per cent confidence limits were determined from a probit-log dose regression line calculation as described by Finney.[27] In antagonism studies, the choline chloride was given by i.p. administration immediately after the test drug had been injected.

Cat sciatic nerve–gastrocnemius muscle preparation. Cats of either sex weighing between 2·5–3·5 kg were anesthetized by an i.p. injection of 0·6 ml per kg of Dial with Urethane (Ciba Pharmaceutical Company). The trachea was cannulated with a "Y" tube and the animal was allowed to respire naturally. Artificial respiration was provided when necessary by means of a Harvard respiration pump. The sciatic nerve was exposed by making an incision on the posterior side of the thigh and retracting the underlying muscles. The nerve was then dissected free from surrounding tissue, tied, and crushed proximally. A bipolar, shielded, silver electrode was then placed on each sciatic nerve. The Achilles tendon was severed distally and attached to a Grass FT 10 force-displacement transducer. The leg was immobilized by placing a stainless steel

rod through the knee joint. The rod was then firmly attached to a metal frame. The foot was secured to the frame by means of a clamp. All drugs were routinely examined for frequency-dependency in bilateral preparations. The nerve of one muscle was stimulated at 1 c/s, and the nerve of the contralateral muscle was stimulated at 0·1 c/s. Supramaximal voltage with a pulse duration of 0·3 msec was delivered by a Grass S4 stimulator. Compounds were dissolved in physiological saline solution and injected i.v. by means of a cannula inserted into the left jugular vein. Blood pressure was measured in this preparation by means of a cannula inserted into the right carotid artery and connected to a Statham P23 AC blood pressure transducer. Blo od pressure and muscle twitches were recorded simultaneously on a Grass polygraph (model 5).

Cat tibialis anterior muscle preparation (for close-arterial injections). A modification of the method of Brown[28] was used. Cats of either sex weighing between 2·5 and 3·5 kg were anesthetized by an i.p. injection of 0·6 ml. per kg of Dial with Urethane. One leg was secured to a supporting framework by means of a nail driven through the head of the tibia and a clamp placed on the tibia near the ankle. The distal tendon of the tibialis was dissected free from its attachment together with a fragment of bone and tied to a Grass FT 03 force-displacement transducer by a small metal rod. The preparation of the sciatic nerve for stimulation was as mentioned above. The nerve was stimulated supramaximally at 1 c/s with a pulse duration of 0·5 msec. Retrograde administration (close-arterial) of ACh chloride in a volume of 0·15 ml was made through a small-bore cannula inserted in the tibial artery. The popliteal artery was not occluded during the close-arterial injections of ACh. A jugular vein was used for the i.v. administration of compounds.

Measurement of ganglionic blockade in the cat. Drug-induced block of ganglionic transmission was measured *in situ* using the superior cervical ganglion of the cat as described by Volle[29] and by Takeshige and Volle.[30] Supramaximal stimuli at 0·5 c/s (0·25 msec duration) were applied to the cervical sympathetic trunk by means of a square-wave generator, the output of which was passed through a stimulus isolation unit to bipolar platinum electrodes. One electrode was placed in direct contact with the surface of the ganglion, the other on the crushed end of the postganglionic nerve. The evoked potentials were amplified by a resistance-coupled preamplifier and visualized on an oscilloscope. Permanent records were made on moving photographic paper. Drugs were administered through a 27-gauge needle which was inserted into the common carotid artery and clamped to the supporting framework. The volume of injection was 0·1 ml. All of the drugs were dissolved in physiological saline. Clotting in the needle was prevented by the prior administration of heparin (300 units/kg i.v.).

Measurement of acetylcholinesterase inhibition. The determination of acetylcholinesterase (AChE) inhibition *in vitro* was made by the manometric technique originally described by Ammon and later modified by Augustinsson.[31] The total volume in the reaction flask was 2·0 ml. ACh chloride at 5×10^{-3}M was used as the substrate. Readings of CO_2 evolution were taken every 10 min for 1 hr. The enzyme was true cholinesterase from bovine erythrocytes (Sigma Chemical Co.). At least three different concentrations of each compound were used to obtain I_{50} values. Three determinations with duplicate samples were made for each compound.

Biosynthesis of ACh by minced brain. For each experiment a 400–500 g male guinea pig was stunned and the brain was quickly removed and rinsed with Locke solution. The cold tissue was then placed in a plastic tube closed at one end by a plunger and

at the other end by two layers of fine nylon stocking. The mince was prepared by slowly extruding the tissue through the nylon mesh. Samples of mince weighing between 130 and 150 mg were suspended in ice-cold modified Locke solution consisting of NaCl (130 mM), $CaCl_2$ (2 mM), KCl (4 mM), $NaHCO_3$ (25 mM), eserine sulphate (20 μM), and the desired concentration of test substance. The total volume of medium for each sample prior to the addition of tissue was 3·0 ml. The medium and mince were placed in 20-ml beakers to permit efficient agitation and oxygenation. Duplicate samples were used. Two control samples, containing no test substance, were made up to volume by additional medium. Two samples similarly prepared but not incubated were used for estimation of free ACh at zero time. Incubation was for 75 min at 37°. Throughout this period the beakers and their contents were gently agitated on a Dubnoff shaker and kept under moist O_2–CO_2 (95:5) so that the pH of the medium was 7·5 $\pm$ 0·1. At the end of incubation, the mince suspensions were quickly chilled and centrifuged at 4° for 20 min at 3300 rpm. The supernatant was decanted from each sample and bioassayed immediately on the guinea pig ileum[32] or the rat blood pressure.[33] The extraction of bound ACh from tissue residues was done according to Mann *et al.*[34] The extracts were bioassayed on the guinea pig ileum after adjustment to pH 4·5 and overnight storage at 4°. Bioactivity was considered to be due to the presence of ACh by the following criteria: (1) activity could be abolished by alkaline-boiling (pH 11) the sample; (2) activity could not be abolished by acid-boiling (pH 4) the sample; (3) Activity could be abolished by atropinization of the bioassay preparation; (4) activity could be abolished by excluding eserine from the incubation medium.

RESULTS

Comparison of compounds I, II, and III with HC-3

Table 1 summarizes some of the properties of compounds I-III. All three analogs had a maximal absorption in the ultraviolet spectrum at 257 mμ. The infrared spectra of these products showed very weak or no carbonyl absorption at 5·9 μ. These data confirm that the compounds resemble HC-3 in that they exist predominantly in the hemiacetal form.

Symptoms of toxicity in mice produced by each compound were identical to those produced by HC-3. Death occurred in 15–20 min and was apparently due to respiratory paralysis. The toxic syndrome and fatality produced by a near-LD_{90} dose of each compound was abolished by injecting choline chloride (20 mg/kg i.p.) immediately after administration of the test drug. Compound I, bearing methyl substitutions at the α'-positions, was equipotent with the parent molecule. Methyl substitutions on the β-ethanol side chains resulted in less active products: HC-3 was roughly 2·5 and 8 times more toxic than compounds II and III respectively. No member of this series was a potent inhibitor of acetylcholinesterase *in vitro*. As shown in Table 1, high concentrations ($>10^{-4}$M) were necessary to reach I_{50} level. The I_{50} values of neostigmine methyl sulfate and eserine sulfate under the same experimental conditions were, respectively, $7{\cdot}2 \times 10^{-8}$M and $6{\cdot}1 \times 10^{-8}$M.

The relative activities of compounds I and III as inhibitors of neuromuscular transmission were similar to the relative activities observed in LD_{50} determinations. Compound I was equiactive with HC-3 in the cat sciatic nerve–gastrocnemius muscle preparation (stim. freq., 1 c/s). At 150 μg/kg, HC-3 and compound I produced a

gradual diminution of twitch height, reaching a maximal depression in 45–60 min. Increasing this dose 5-fold did not increase the degree of depression. Compounds II and III were inactive in this preparation at 150 μg/kg. About two to three times as much compound III was necessary to initiate observable depression as compound II. At 750 μg/kg, all analogs produced hemicholinium-like activity with little difference in magnitude of depression of twitch height (Fig. 2). In all experiments

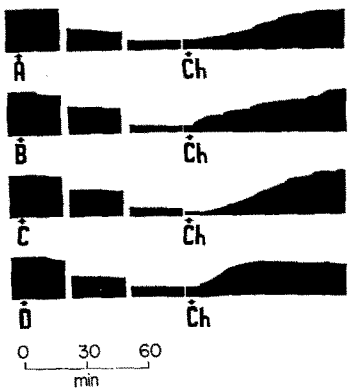


FIG. 2. Maximal twitches of the gastrocnemius muscle of the cat elicited by single-shock stimulation of the sciatic nerve once every sec. At the arrows, 750 μg/kg of compound was injected intravenously. A, HC-3; B, compound I; C, compound II; D, compound III. When maximal depression of twitch height was reached, 1 mg/kg of chlorine chloride (Ch) was injected intravenously every 100 sec until antagonism was complete.

neuromuscular inhibition was antagonized by the administration of choline chloride as shown in Fig. 2. Compound II at a dose of 1 mg/kg produced an initial transient depression of twitch height prior to proceeding into a hemicholinium-like blockade. Paralysis brought about by any of the compounds at these low doses was frequency-dependent: contractions of the slowly stimulated contralateral muscle (stim. freq., 0·1 c/s) in bilateral preparations remained maximal. Only compound II modified the blood pressure of these animals; at 750 μg/kg, a slight hypotension, lasting 5–10 min, was produced.

Separation and confirmation of seco- and hemiacetal-IV

For synthesis of compound IV, the appropriate amino-alcohol precursor was used that would introduce two methyl groups at each α-position of the HC-3 molecule. Examinations of the product in water or ethanol (95%) in the ultraviolet spectrum revealed a second maximum at about 285 mμ in addition to the characteristic maximum at 257 mμ for the hemicholiniums previously mentioned. The infrared spectrum of compound IV showed strong carbonyl absorption. The ultraviolet absorption at 285 mμ and the infrared carbonyl absorption declined progressively with each recrystallization; 4–5 recrystallizations of compound IV from ethanol–ether yielded a product virtually isochromic with HC-3 in both the infrared and ultraviolet spectra. Paper chromatography of the unrecrystallized compound IV revealed two well separated

spots ($R_f = 0{\cdot}62$ and $0{\cdot}86$). Compound IV, which was subjected to repeated recrystallization, gave only a single spot with an R_f of $0{\cdot}62$. The toxic syndrome produced in mice by the product mixture IV (unrecrystallized) was similar to that of HC-3. The LD_{50} which at first was 2–3 times that of HC-3, gradually decreased with each subsequent recrystallization and eventually became equal to that of HC-3. These data suggested that the original compound IV mixture might have as components stable ring-chain tautomers (Fig. 3).

The relative amounts of the tautomers of IV, opened-ring (seco)-IV, and closed-ring (hemiacetal)-IV were found to depend on the amount of heat imparted to the original reaction of α,α'-dibromo-4, 4′-biacetophenone with 2-dimethylamino-2-methyl-1-propanol. As shown in Fig. 4, when the reaction was allowed to stand overnight at

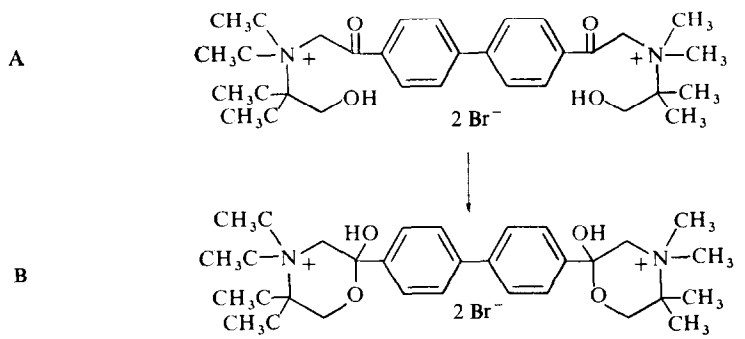


FIG. 3. Conversion of seco-IV (A) to hemiacetal-IV (B).

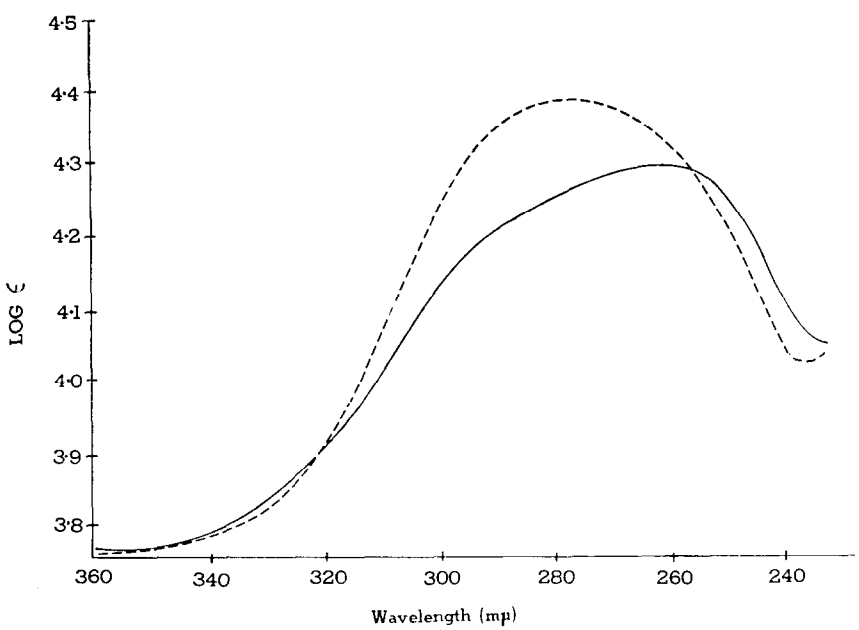


FIG. 4. Ultraviolet absorption curves of compound IV. The broken line is the spectrum of the compound prepared at room temperature. The continuous line is the spectrum of the compound prepared by allowing the reaction mixture to remain at reflux temperature (about 70°) for 1½ hr.

room temperature, a greater proportion of seco-IV resulted, as indicated by the increased bathychromic absorption in the ultraviolet spectrum. When the reaction was stirred at reflux temperature for ½ hr, more hemiacetal-IV resulted, as indicated by the increased hypsochromic absorption. This hypsochromic shift produced by elevated temperatures would be expected if the compound were being converted to a less conjugated molecule. For compound IV, the more conjugated seco-IV would be converted to the less conjugated hemiacetal-IV. An alternate mechanism would be simultaneous quaternization and hemiacetalization. An increase in the temperature would aid the formation of the transition state required by such a mechanism. Since

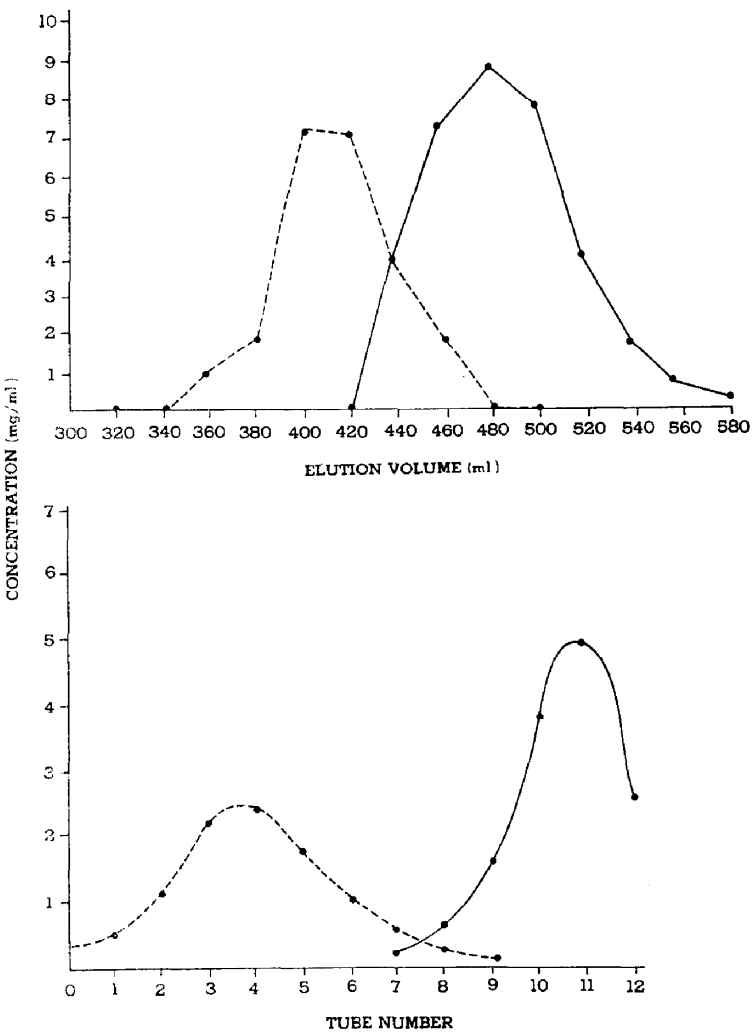


FIG. 5. The separation of seco-IV from hemiacetal-IV. Upper panel: elution diagrams with *n*-butanol-ethylene glycol–water (4:1:1) from fibrous cellulose column. Lower panel: countercurrent distribution analysis of compound IV. The curves represent fitted theoretical binomial distributions. For both separation methods, 1 g of IV was used. · – – – ·, seco-IV; ·———·, hemiacetal-IV. The *K* (*n*-butanol–H_2O) values for seco- and hemiacetal-IV were 1·94 and 0·11 respectively.

these tautomers were able to exist independently of each other, they qualified as desmotropes.

The results of the separation of seco- and hemiacetal-IV are shown in Fig. 5. Countercurrent distribution as well as a cellulose column were used as methods to separate the desmotropes. Seco-IV was considerably surface-active and produced stable emulsions in tubes 0 through 7 of the countercurrent distribution. This property was not observed for hemiacetal-IV, which distributed mainly in tubes 9 through 12. Inasmuch as less overlap of the desmotropes was achieved by countercurrent distribution, this method was used routinely for separations. With either preparative method of separation, only two components of compound IV were apparent. This is in good agreement with the two iodoplatinate-positive spots detected on chromatograms of compound IV. The ultraviolet spectrum of the seco component isolated from tube 3 had a λ_{max} at 292 mμ (Fig. 6.) The more substantial confirmation of the seco- and hemiacetal assignments was afforded by infrared spectroscopy (Fig. 7). The

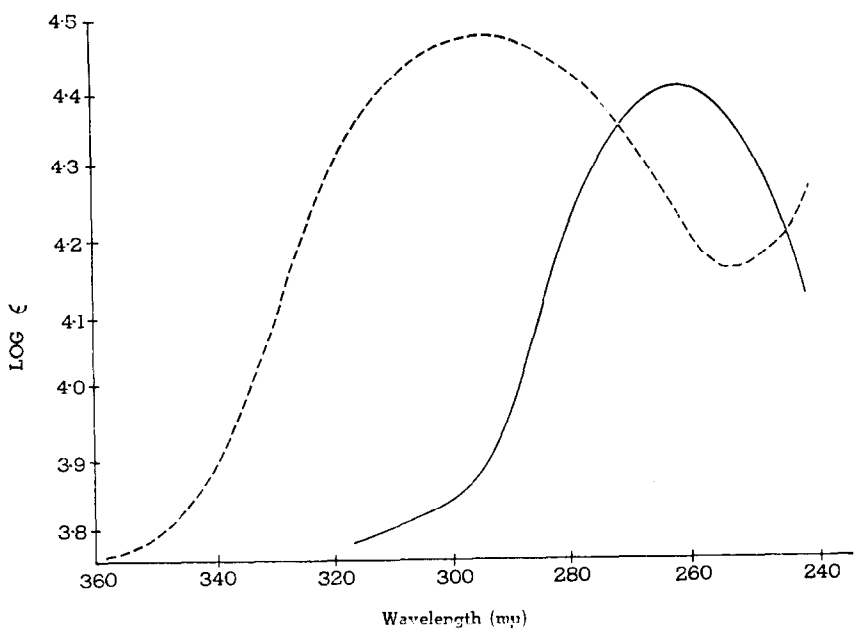


FIG. 6. Ultraviolet spectra of isolated seco-IV (- - - -) and hemiacetal-IV (———).

most dramatic differences are to be seen in carbonyl absorption. The tube 3 product (seco-IV) showed an intense carbonyl absorption (5·9 μ) that was practically absent in the tube 12 (hemiacetal-IV) product. Hemiacetal-IV showed strong absorption at 7·6 μ, which was not observed for seco-IV. This absorption was attributed to OH in plane deformation.[35] Full acetal derivatives of HC-3, in support of this OH functional assignment, did not have any absorption at this wavelength (F. Bove, personal communication). The infrared spectrum of seco-IV eluted from a cellulose column with chloroform–methanol (10:1) was superimposable on that of seco-IV separated by countercurrent distribution. Proton nuclear magnetic resonance spectra of seco- and

hemiacetal-IV (Fig. 8) were also compatible with the structures shown in Fig. 3. Table 2 summarizes some of the properties of the isolated desmotropes of compound IV. Note that for both thin-layer and paper chromatograms the R_f of isolated seco-IV coincided with that of the higher R_f component of the unseparated desmotropic mixture (seco-IV and hemiacetal-IV), while the R_f of isolated hemiacetal-IV coincided with that of the lower R_f component of the desmotropic mixture.

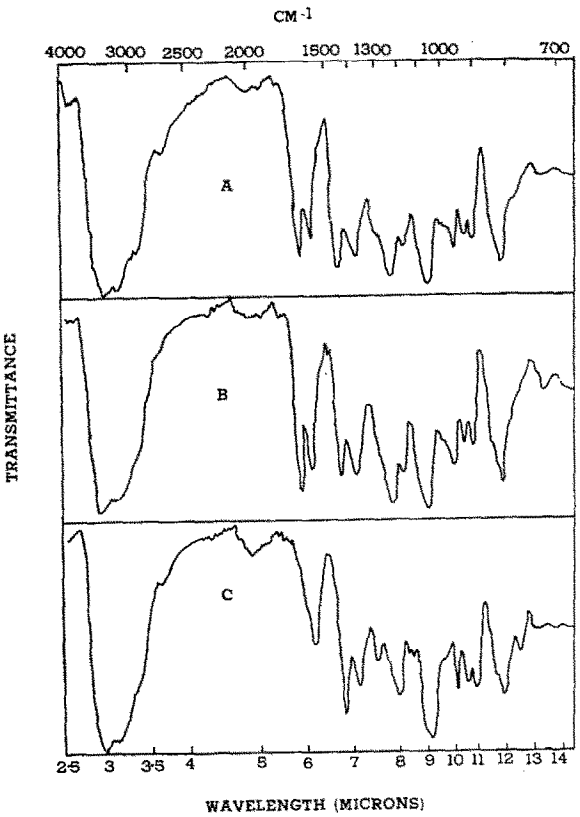


FIG. 7. Infrared spectra of compound IV (A) as the unseparated desmotropic mixture, seco-IV (B) and hemiacetal-IV (C).

Biological activities of seco- and hemiacetal-IV

The lack of rapid spontaneous ring-closure, or hemiacetalization, of seco-IV in aqueous solutions to form hemiacetal-IV, and vice versa, made possible a comparison of the pharmacology of these compounds. Solutions of seco-IV in physiological saline which were allowed to remain at room temperature for up to 6 months, exhibited at most only a 10 mμ hypsochromic shift in the ultraviolet spectrum compared with fresh solutions. Refluxed (5 hr) ethanol solutions of seco-IV did not give any spectral evidence of hemiacetalization. Hydrogen bonding with water might be stabilizing seco-IV so that its β-OH groups are kept from approaching the carbonyl functions.

Solutions of hemiacetal-IV under any of these conditions did not change from a λ_{max} of 257 mμ. The conversion of hemiacetal-IV to seco-IV in these solutions would have been manifested by a bathychromic shift in the ultraviolet spectrum. Acidification (HCl) of aqueous solutions did not produce any marked spectral changes, but basification (KOH) of seco-IV (pH 9) caused a hypsochromic shift. Under severe alkaline conditions (pH 12) seco-IV has a λ_{max} of 261 mμ. This shift could not be reversed by reacidification. True internal hemiacetalization is doubted to have taken

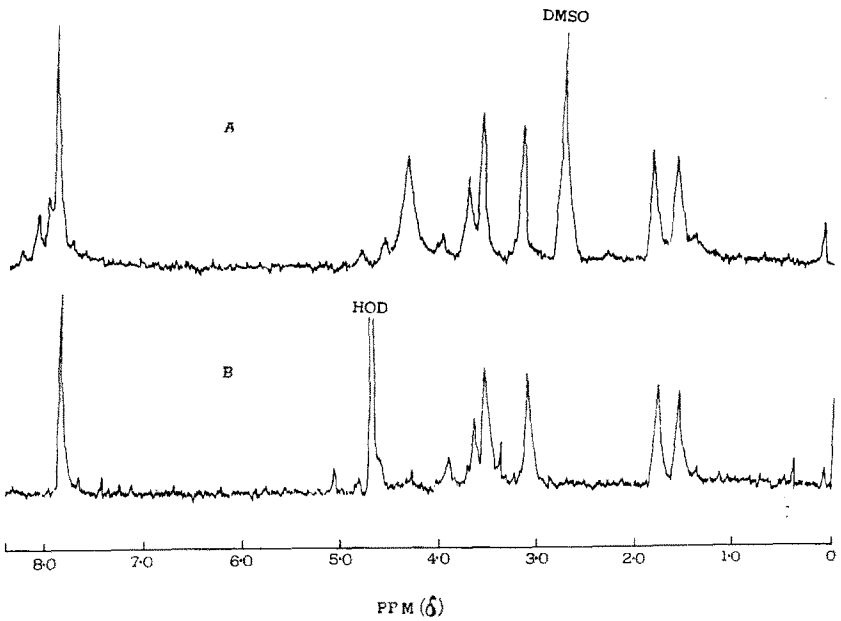


FIG. 8. Proton n.m.r. spectra of seco-IV (A) and hemiacetal-IV (B). Seco -IVwas dissolved in dimethylsulfoxide (DMSO-d_6) and heated to 50% to maintain an adequate concentration in solution. The shifted water peak at 4·3 ppm in spectrum A is attributed to these solvent-temperature effects. D_2O at room temperature was a suitable solvent for hemiacetal-IV.

place, since neutralized solutions of the base-converted product did not have the biological properties of hemiacetal-IV. Alkaline treatment of hemiacetal-IV brought about no more than a 3 mμ bathychromic shift, which was reversed by reacidification.

In acute toxicity studies, hemiacetal-IV was equipotent with HC-3 and at least 200 times more potent than seco-IV in producing fatality in mice (Table 2). Both desmotropes produced a toxic syndrome in the animals similar to that of HC-3; the syndrome and fatality in mice produced by near-LD_{90} doses were effectively antidoted by choline chloride (20 mg/kg i.p.). It could be possible that the hemicholinium-like activity observed in mice for seco-IV was not due to the compound as such, but instead, was due to a slight contamination of hemiacetal-IV.

Both desmotropes require high I_{50} concentrations for acetylcholinesterase. It is interesting that the unseparated desmotropic mixture should have a lower I_{50} value than either of its components (Table 2).

The desmotropes were examined in the sciatic nerve–gastrocnemius muscle preparation of the cat. With single-shock stimulation at a frequency of 1 c/s, seco-IV (750 μg/kg i.v.) did not reduce twitch height, but caused a large decrease in blood pressure (Fig. 9). The severe hypotension produced in this preparation by seco-IV prevented studies with high doses. Atropine and diphenhydramine given at various doses prior to injection of seco-IV did not protect animals from a sustained fall in blood pressure. As will be indicated below, this hypotension was probably due to a blockade of ganglionic transmission. Hemiacetal-IV was extremely active in nerve–muscle studies,

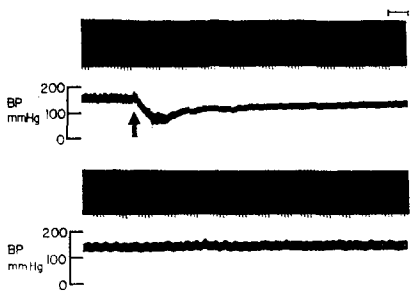


FIG. 9. Cat sciatic nerve–gastrocnemius muscle preparation. Maximal twitches were elicited by single-shock stimulation of the sciatic nerve once every sec. BP denotes carotid blood pressure. At the arrow, 750 μg/kg of seco-IV was given intravenoulsy. The interruption in the record is 40 min; the horizontal calibration is 1 min.

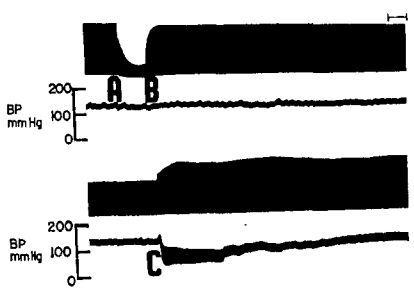


FIG. 10. Cat sciatic nerve–gastrocnemius muscle preparation. Maximal twitches were elicited by single-shock stimulation of the sciatic nerve once every sec. BP denotes carotid arterial blood pressure. A, hemiacetal-IV (250 μg/kg i.v.); B, neostigmine methylsulfate (50 μg/kg i.v.); C, choline chloride (4 mg/kg i.v.). The interruption in the record is 40 min; the horizontal calibration is 1 min.

both as a curare-like and as a hemicholinium-like agent. As shown in Fig. 10, hemiacetal-IV, at a dose of 250 μg/kg i.v., produced a precipitous reduction of twitch height that could be completely antagonized by neostigmine methylsulfate (50 μg/kg i.v.); after this antagonism, a second but more slowly developing hemicholinium-like neuromuscular depression occurred that could be completely reversed with choline chloride (4 mg/kg i.v.). When the dose of hemiacetal-IV was lowered to 150 μg/kg, only the late-phase, or hemicholinium-like blockade was observed. Neostigmine at best could only partially reverse the late-phase blockade. With muscles receiving indirect

stimulation at a lower frequency (0·1 c/s), only the early-phase blockade could be demonstrated even when the dose of hemiacetal-IV was increased to 1 mg/kg. It is important to mention at this point that in nerve–muscle and blood pressure studies, like those described above, compound IV in the form of unseparated desmotropes acted qualitatively as though it were a chemical composite of seco- and hemiacetal-IV.

To substantiate that a prejunctional (hemicholinium-like) as well as a postjunctional (curare-like) blockade of neuromuscular transmission was occurring with hemiacetal-IV, the cat tibialis anterior muscle was prepared for close-arterial injections of acetylcholine. The results are shown in Fig. 11. The biphasic pattern of inhibition is clearly

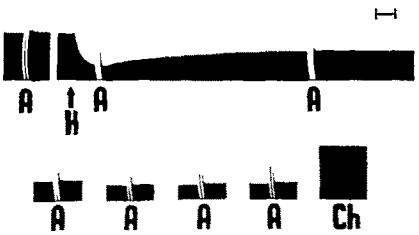


FIG. 11. Maximal twitches of the cat tibialis anterior muscle elicited by single-shock stimulation of the sciatic nerve once every sec. At H, 250 μg/kg of hemiacetal-IV was given intravenously. At A, electrical stimulation was temporarily stopped and 5 μg acetylcholine chloride was injected close-arterially. Ch represents the maximal antagonism of neuromuscular blockade achieved by giving 1 mg choline chloride intravenously every 100 sec. The breaks in the record represent 20 min; the horizontal calibration is 1 min.

shown in this figure. After the administration of hemiacetal-IV (250 μg/kg i.v.), the tension of contractions produced by sciatic nerve stimulation rapidly became weaker, then returned partially for a few minutes, and finally fell into a second phase of inhibition. The contractures produced by close-arterial injections of 5 μg ACh chloride on the unstimulated muscle were completely abolished for approximately 40 min after hemiacetal-IV. This observation supports a postjunctional inhibition for the early phase blockade. During the late-phase blockade, the contractures induced by exogenous ACh became prominent even in the presence of a 75 per cent reduction in twitch tension. Since the effector area of the muscle could still respond to exogenous acetylcholine, the evidence here is in favor of a prejunctional rather than a postjunctional site of action for the late-phase blockade.

Hemiacetal-IV was much less active than seco-IV in reducing blood pressure. This same relative activity of the desmotropes was also observed in blockade of transmission in the superior cervical ganglion of the cat. As shown by the results of the experiment illustrated graphically in Fig. 12, seco-IV injected close-arterially at a dose of 20 μg produced a maximal 40 per cent blockade of postganglionic nerve potentials evoked by stimulation of the preganglionic nerve. A blockade of smaller magnitude was produced by 100 μg of either hexamithonium dichloride or hemiacetal-IV. Note that in the second tracing of this figure 100 μg seco-IV virtually nullified transmission. HC-3 at a dose of 200 μg produces a small transient depression of spike height accompanied by a slight depolarization (A. D. Winters, personal communication).

Further differentiation of the hemicholinium-like activities of seco- and hemiacetal-IV was obtained when these compounds were examined for their ability to interfere with acetylcholine synthesis *in vitro*. Figure 13 shows the absolute amounts of ACh released into the medium (free ACh) during the incubation of guinea pig minced brain. The synthetic activity observed in those samples without any inhibitor present was slightly less than that reported by Bhatnagar and MacIntosh[36] for minced brain tissue of mice. At 10^{-4}M, HC-3, seco- and hemiacetal-IV strongly inhibited ACh synthesis to almost the same extent. However, at 10^{-5}M, the inhibition produced by seco-IV

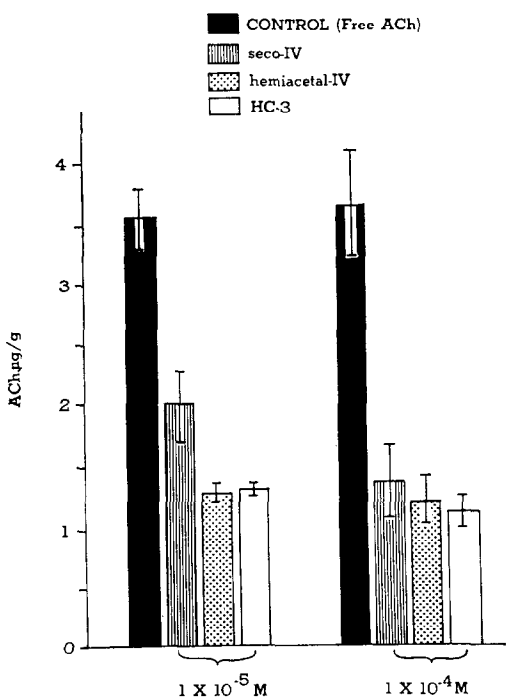


FIG. 13. Inhibition of acetylcholine (free) synthesis in guinea pig minced brain by HC-3, seco-IV and hemiacetal-IV. Each bar represents the mean of 6 experiments ± S.D. Each beaker contained 130–150 mg (wet weight) of brain mince in eserinized bicarbonate–Locke solution (pH 7·5 ± 0·1) and was agitated on a Dubnoff shaker at 37° for 75 min under an atmosphere of 95% O_2: 5% CO_2.

was significantly less than that of either hemiacetal-IV or HC-3. To show that low levels of ACh in the medium were indicative of suppressed synthesis and not release, an estimation was made of "bound" ("combined") ACh in the tissue residue after incubation. In four trials, one for each drug concentration, the levels of free AC roughly paralleled the much higher levels of bound ACh. Therefore, the depression of formation of free ACh was a reflection of inhibition of ACh biosynthesis.

DISCUSSION

Three methyl analogs of HC-3 (compounds I, II and III) were shown from spectrophotometric evidence to undergo hemiacetalization as described originally by Schueler[9] for the parent structure. These compounds were shown to possess hemicholinium-

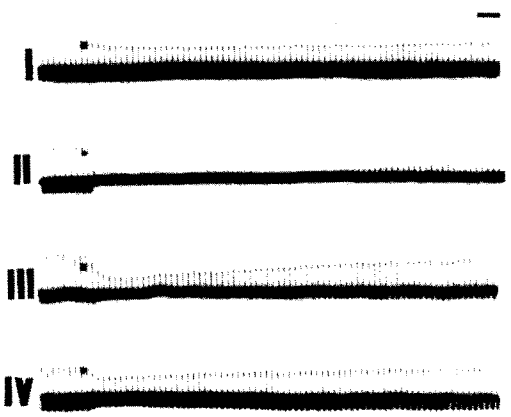


FIG. 12. Postganglionic nerve potentials evoked by supramaximal stimulation (single-shock) of the cat preganglionic cervical sympathetic trunk at a frequency of 0·5 c/s. The vertical calibration is 1 mV and represents the time of injection. I, hexamethonium dichloride [100 μg intra-arterially (i.a.)]; II seco-IV (100 μg i.a.); III, seco-IV (20 μg i.a.);IV, hemiacetal-IV (100 μg i.a.). The horizontal calibration is 4 sec.

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like activity. Only compound I was equipotent with HC-3. Compounds II and III exhibited about a 2- and 8-fold potency decrease respectively. These data suggest that the two choline moieties of HC-3 that bear the α- and β-positions (see Fig. 1) are crucial for competing with choline in an active transport process at nerve terminals. However, this crucial moiety was not given support by hemiacetal-IV, since this compound was equipotent with HC-3 while bearing methyl substitutions on the α-positions. Compounds II and III were also examined by Long *et al.*[20] and were found to have biological properties coinciding qualitatively and quantitatively with those reported in this study.

After studying 19 congeners of HC-3, Schueler[9] stated that only those compounds which possessed the "unique chemical peculiarity" of hemiacetalization had activity resembling HC-3. He found that only phenacyl*ethanol*amines undergo this type of cyclization, which was evidenced by a great depression of carbonyl absorption in the infrared spectrum as well as by a hypsochromic shift in the ultraviolet spectrum. When the β-hydroxyl groups were replaced by β-ethoxy groups (see Fig. 14A), hemiacetalization as well as hemicholinium-like activity was absent. Hemicholinium-like

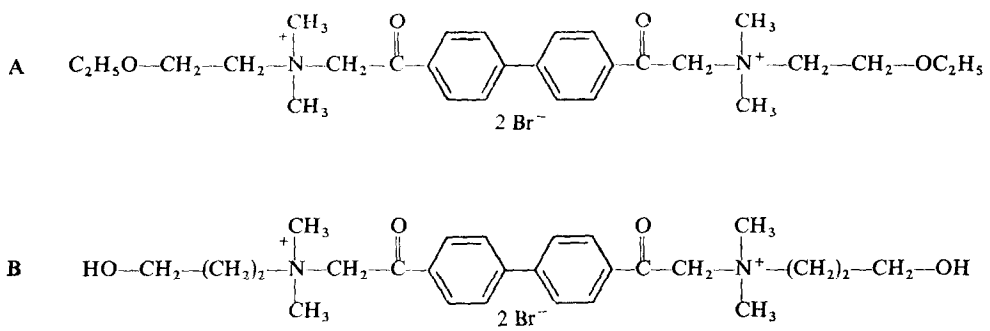

FIG. 14. Non-hemiacetal (seco) congeners of HC-3.

properties could not be shown in other derivatives where steric hindrance and restricted rotation precluded hemiacetalization. Domer and Schueler[37] could not detect any biotransformation of C^{14}-labeled HC-3 in rats 24 hr after intraperitoneal administration, ruling out a metabolite as an "active" structure. Also, it would have been quite likely that had the HC-3 given to these rats "unlocked" its cyclic hemiacetal structure, the resulting seco- (opened-ring) HC-3, *if stable* would have been detected in the urinary radiochromatograms. V. B. Haarstad and F. W. Schueler (unpublished data) have provided direct evidence of the instability of seco-HC-3 and the persistence of hemiacetal-HC-3. They have shown that HC-3 can be prepared in a manner such that it can exist prior to recrystallization as the seco form, provided that the compound remains in a relatively anhydrous environment. In aqueous and alcohol solutions, this seco-HC-3 rapidly and completely cyclized to form hemiacetal-HC-3. They also found that HC-3 in aqueous solutions was extremely stable, maintaining for over 2 years the same ultraviolet spectrum, and activity in mice, as the freshly prepared solution. Highly acidic or basic solutions failed to convert HC-3 to its seco form. The above observations favor the hemiacetal form of HC-3 as its active structure.

The separation of compound IV into two components by countercurrent distribution has provided a system by which a hemiacetal form of a hemicholinium analog could be compared with its corresponding seco form. In essence, compound IV has served as a model for HC-3 by supplying both tautomers. A comparison was allowed by the fact that one of the desmotropes (hemiacetal-IV) was equiactive with HC-3. The absolute purity of the separated recrystallized seco IV-remains to be ascertained (cf. Table 2). Appropriate elemental analysis was obtained for the desmotropic mixture, however. It is possible that seco-IV decomposes slightly during isolation, although chromatographic and spectrophotometric comparisons of seco-IV before and after separation do not indicate the presence of a second component. Furthermore, all the pharmacological responses of the isolated seco-IV were also observed for the analytically pure desmotropic mixture. The failure to obtain satisfactory elemental analyses could be due to strong retention of alcohol and water by the recrystallized seco-IV.

In this study the following criteria were used to demonstrate hemicholinium-like activity: (1) failure of respiration in mice, terminating in death within 10–20 min, with choline serving as an antidote; (2) a slow progressive inhibition of neuromuscular transmission in cats, which occurred only at high frequencies of stimulation, and which was antagonized by choline; (3) an inhibition of minced-brain biosynthesis of ACh. By these criteria, seco-IV possessed only a weak hemicholinium type of activity. Seco-IV did show marked inhibition of transmission in the superior cervical ganglion of the cat. This ganglionic blockade could be the mechanism for the hypotensive effect observed and may arise from a reduced interquaternary distance. Hemiacetal-IV would maintain a greater interquaternary distance because of its stable ring structures. Mertes *et al.*[38] have recently suggested that potent ganglionic blockade could result in compounds having a distance in the range of 4·5 to 5·5 Å between the quaternary heads. Dreiding models of HC-3 provided an estimated interquaternary distance of 14 Å, which is in agreement with the low ganglionic blocking activity of HC-3.

Hemiacetal-IV qualified in all evaluations as a hemicholinium type of compound and was apparently just as active as HC-3. In nerve–muscle studies, this desmotrope also produced a curare-like blockade. Long *et al.* [20]also prepared compound IV, but their data indicate that they were working primarily with the hemiacetal form of this compound. Most or all of the seco material probably was removed by repeated recrystallization.

It is obvious now that other derivatives of HC-3 can be made to elaborate stable seco and hemiacetal desmotropes. Schueler[9] reported that the structure shown in Fig. 14B—not being a phenacyl*ethanol*amine—would be unlikely to form the 7-membered oxazepinium rings, but later, Anderson *et al.*[39] showed that this ring formation can take place under more favorable reaction conditions. Thus, by using two different reaction conditions, desmotropes can be prepared without having to resort to tedious separation procedures. Infrared and ultraviolet spectroscopy would be excellent tools to establish confirmation of structure for these desmotropes.

In giving any consideration to a structure–activity relationship of HC-3, it is important to note that the hemiacetal structure is not obligatory for inhibition of ACh biosynthesis. Triethylcholine and FWH 429, or triethyl-2(3, 4, 5-trimethoxybenzoyloxyethylammonium) tosylate were also found to resemble HC-3 *in vivo*; these compounds were also able to reduce the synthesis of ACh in respiring brain mince

tissue.[7, 40, 41] The relative activities both *in vivo* and *in vitro* were in decreasing order HC-3 > FWH 429 > triethylcholine. Benz and Long[42] made a study of the spatial requirements for HC-3 and proposed that the cyclic hemiacetal was unnecessary for hemicholinium-like activity. Though these congener and non-congener structures may be active as inhibitors of ACh biosynthesis, the present results maintain the thesis that, for HC-3 *per se*, the hemiacetal structure is requisite for such inhibition and probably compliments exceptionally well the rate-limiting choline "carrier" system at nerve terminals.

In a more general sense, the present results are consistent with the fact that quaternary bases can affect cholinergic transmission in a number of ways. The pharmacology of hemiacetal-IV shows that such bases can simultaneously attack both presynaptic and postsynaptic sites, indicating that at these sites there may be similar drug–receptor interactions. In addition, this model study has established that isolatable tautomers can evoke different biological responses, just as geometrical and optical isomers do.

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