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ACETYLSECO HEMICHOLINIUM-3, A NEW CHOLINE ACETYLTRANSFERASE INHIBITOR USEFUL IN NEUROPHARMACOLOGICAL STUDIES*

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Summary—Described are the synthesis and some aspects of the pharmacology of acetylseco hemicholinium-3 (acetylseco HC-3), the acetylated open ring analogue of hemicholinium-3 (HC-3). The effects of both compounds were determined *in vivo* on rat brain acetylcholine (ACh), ^{14}C-choline (^{14}C-Ch) incorporation into ^{14}C-acetylcholine (^{14}C-ACh) and on one way jump box avoidance and escape behavior in naive and trained rats. In addition, the *in vitro* effects of both drugs were determined on choline acetyltransferase activity (ChAc) in rat brain.

When given intraventricularly in doses of 1–20 μg both compounds reduced total ACh content in the brain to a maximum of 50% of normal in 30–60 min. In doses of 20 μg intraventricularly, both drugs also reduced ^{14}C-Ch incorporation into ^{14}C-ACh by 84·5% for acetylseco HC-3 and by 52% for HC-3.

The *in vivo* changes of ACh in the brain were correlated with the behavioral deficits induced in one way shuttle box acquisition and retention. In doses of 20 μg total intraventricularly, both compounds produced behavioral deficits which were greater in naive than in trained animals. *In vitro*, acetylseco HC-3 inhibited ChAc activity with an I_{50} of 1×10^{-5} M with Ch 10^{-2} M and acetyl CoA $6{\cdot}4 \times 10^{-4}$ M, while HC-3 had no inhibitory effects. Using rat brain homogenate as the enzyme source and commercial acetyl CoA for kinetic studies, acetylseco HC-3 was shown to be a mixed inhibitor of acetyl CoA and a competitive inhibitor of Ch.

The *in vivo* actions of acetylseco HC-3 are consistent with those of a ChAc inhibitor. However, it is necessary to rule out the possibility that the drug may also compete with Ch for its transport across biological membranes like its deacetylated derivative HC-3.

The hemicholiniums have been widely investigated since they were first synthesized in 1954 by LONG and SCHUELER. Hemicholinium-3 (HC-3) is the prototype compound of this series. This agent reduces tissue acetylcholine (ACh), possibly by reducing the active transport of choline (Ch) as shown by MACINTOSH (1963) and HODGKIN and MARTIN (1965). However, other mechanisms such as a shift in Ch metabolism toward phospholipid formation (GOMEZ, DOMINO and SELLINGER, 1970a,b; GOMEZ, SELLINGER, SANTIAGO and DOMINO, 1971) and production of a false neurotransmitter through acetylation (RODRIGUEZ DE LORES ARNAIZ, LIEBER and DE ROBERTIS, 1970) have been proposed. SCHUELER (1955) found that HC-3 was the most toxic of some 20 bis-quaternary derivatives studied. HC-10 or acetylseco hemicholinium-3 (acetylseco HC-3) produced a toxicological picture similar to HC-3. SCHUELER assumed that acetylseco HC-3 was hydrolyzed *in vivo* to HC-3. There was little evidence then that acetylseco HC-3 was pharmacologically different from HC-3.

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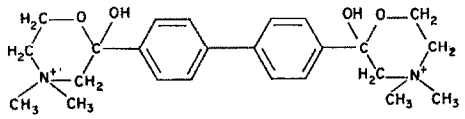
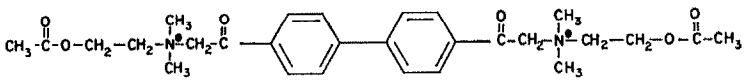
The chemical structures of these two compounds are given in Figure 1. Note that HC-3 exists in the hemiacetal form. The fact that "hemiacetal" formation in the ring of HC-3 is not essential for neuromuscular blocking effects (GESLER, LASHER, HOPPE and STECK,



Hemicholinium -3



Acetylseco HC-3

Fig. 1. Comparison of structures of acetylseco HC-3 and HC-3.
Note that both compounds are bisquaternary nitrogen derivatives. HC-3 exists as the hemiacetyl ring structure.

1959) and that acetylseco HC-3, the acetate derivative of the opened ring form of HC-3 cannot exist in the hemiacetal form suggests that acetylseco HC-3 might possess different pharmacological actions.

MAGGIO and HAARSTAD (unpublished observations) have reported critical pharmacological differences between HC-3 and acetylseco HC-3 in which inhibition of choline acetyltransferase (acetyl CoA-choline O-acetyltransferase EC2.3.1.6, ChAc) by the latter is especially prominent (DE BALBIAN VERSTER, HAARSTAD and WHITE, 1968, 1969). The present manuscript describes further some of the pharmacological properties of acetylseco HC-3 which indicate it to be an important new tool to inhibit ACh synthesis in the brain when the drug is given intraventricularly.

METHODS

Chemical synthesis

The compound, α,α-dibromo-4,4′-biacetophenone, which is a precursor for acetylseco HC-3, was prepared according to the method of HAARSTAD and SCHUELER (unpublished observations) which is a modification of the method of LONG and SCHUELER (1954). Acetylseco HC-3 dibromide, [4,4′-diphenylylenebis (2-oxoethylene)] bis [(2-acetoxyethyl) dimethylammonium bromide], was prepared as follows: 0·5 g of α,α-dibromo-4,4′-biacetophenone was dissolved in 50 ml of boiling tetrahydrofuran. The solution was allowed to cool to about 50°C. Two ml of freshly distilled 2-dimethylaminoethyl acetate was added all at once while the solution was being swirled. A white precipitate began to form almost immediately. The mixture was allowed to stand at room temperature for 7 hr. The mixture was filtered through a sintered glass funnel. The precipitate was washed well with tetrahydrofuran, then diethyl ether and dried in a vacuum desiccator over anhydrous calcium chloride. Yield 0·7 g (85%) (m.p., becomes an amber glass at 195°, begins to blacken at 205°C. (uncorr.); λ_{max} 305 nm, log E_{max} 4·52; acetate carbonyl, 5·73 μm (s) ketone carbonyl, 5·91 μm (s). Anal. Calcd for $C_{28}H_{38}Br_2N_2O_2$: C, 51·08%; H, 5·82%; N, 4·25%. Found C, 50·91%; H, 5·60; N, 3·99%.

It should be noted that acetylseco HC-3 is prepared analytically pure without the use of a recrystallization step. This has been demonstrated by analyses of several separate preparations. This is to obviate the possibility of transesterification which would lead to the formation of HC-3 itself.

Animal preparations

Male Holtzman rats in groups of 6–12 were used throughout. Both young rats (20–30 days old) as well as adults (90 days and older) were used with intraventricular injection of HC-3 and acetylseco HC-3 in various experiments involving steady state, ACh depletion and ^{14}C-choline (^{14}C-Ch) incorporation into ^{14}C-acetylcholine (^{14}C-ACh). All drug doses were calculated as base. Control solutions of equimolar amounts of NaBr were also used. The young rats were anaesthetized with diethyl ether–air and the bregma exposed to use as a reference point. A puncture point was made on one side approximately 1½ mm posterior and 1½ mm lateral from the bregma to inject either HC-3 or acetylseco HC-3 in the area of the lateral ventricle. A microliter syringe and a needle with a stop were used to inject to a depth of 4–4·5 mm. Rats usually recovered 3–4 min later from anaesthesia and were killed ½ hr later by guillotine. Brains were removed within 45 sec for various assays. Male Holtzman rats 90 days and older were implanted with polyethylene cannulae as described by ROBINSON, HENGEVELD and DE BALBIAN VERSTER (1969) and ALTAFFER, DE BALBION VERSTER, HALL, LONG and D'ENCARNACAO (1970) under pentobarbital anaesthesia. Animals were used 7–14 days after cannulation similar to the young rats except that no anaesthetic was involved on the day of the experiment. Sacrifice occurred 10 min after intraventricular injection of ^{14}C-Ch.

Acetylcholine assay

After sacrifice by decapitation brains were removed, homogenized and ACh extracted by the acidic alcohol method described by STONE (1955) and CROSSLAND (1961). Extracts were bioassayed on the frog rectus abdominus muscle as per DREN and DOMINO (1968). As suggested by FELDBERG (1945), ACh standards were prepared in alkali-inactivated extracts to control for the presence of sensitizing factors in the brain tissue. Although acetone (85%)-1N formic acid (15%) has been reported to be a superior extraction procedure (TORU and APRISON, 1966), it was not used in the bioassay experiments reported because in our hands the frog rectus muscle responded less reproducibly. This extraction procedure was used, however, for the radiochemical studies described below.

Choline acetyltransferase assay

Adult rat brains were used as a source of choline acetyltransferase (ChAc). On the day of the experiment, a rat was decapitated, the brain quickly removed, weighed, and a 10% homogenate prepared with distilled water. The ChAc assay procedure was a modification by DE BALBIAN VERSTER of the method of SCHRIER and SCHUSTER (1967) and MCCAMAN and HUNT (1965). The final concentration of the buffer substrate was: 0·3 M sodium chloride, 0·01 M choline chloride, 2×10^{-4} M physostigmine, 0·02 M magnesium sulphate, 0·05% bovine plasma albumin, 0·08M potassium phosphate (pH 7·4) and $6{\cdot}2 \times 10^{-4}$ M acetyl CoA. ^{14}C-Acetyl CoA was mixed with cold acetyl CoA to yield approximately 20,000 dis/min per incubation tube. Ten μl of the 10% homogenate (1 mg tissue equivalent) were pipetted into each incubation tube. One hundred μl of the substrate were then added with an automatic

pipetter. The tubes were incubated for 30 min at 37°C after which the reactions were terminated by placing the tubes in a boiling water bath for about 5 min. The reaction mixture was then placed onto 0·5 × 8·0 cm columns (diSPo pipets) prepared from prewashed Dowex 1 × 8 chloride. The incubation tubes were then washed with 3 consecutive 0·5 ml water washes, each wash being applied to the Dowex column. The effluent was collected directly into scintillation vials, 15 ml of dioxane scintillation fluid (8·0 g PPO, 0·1 g POPOP, 110 g naphthalene, 1 l dioxane) was added to each vial and the samples counted in a Beckman ambient temperature scintillation counter.

Incorporation of ^{14}C-*choline into* ^{14}C-*acetylcholine*

Unanaesthetized adult male Holtzman rats cannulated at least one week previously were used. Drug and control rats were always done together. Ten min after injection of 20 μl ^{14}C-Ch (1 μCi; 61 mCi/mm) alone or simultaneously with acetylseco HC-3 (20 μg base), the rat was decapitated, the brain quickly removed, weighed, and homogenized in acetone: 1N formic acid (85 : 15). The extraction method of TORU and APRISON (1965) as adapted by SAELENS, ALLEN and SIMKE (1970) was used. After the separation of the organic and H_2O extracts, paper electrophoresis as described by POTTER and MURPHY (1967) and SAELENS *et al.* (1970) was used to separate Ch and ACh in the H_2O extract. The areas were visualized in iodine vapor and the appropriate sections placed into scintillation vials. One ml H_2O and 10 ml of the above described dioxane solution were added to each vial. Counting was done in an ambient temperature Beckman scintillation counter.

One way avoidance behavior

The effects of intraventricular acetylseco HC-3, HC-3, and different equimolar amounts of NaBr for the two compounds were studied on acquisition and retention of rat one way avoidance behavior. The apparatus used was an adaptation of that used by CALDWELL, OBERLEAS, CLANCY and PRAASAD (1970) as described by TENEN (1966). The behavioral parameters were as follows: The conditioned stimulus (CS) was a 5 sec presentation of four 7·5 W red lights with the simultaneous presentation of an escape ledge. At the end of 5 sec, the CS overlapped with a 5 sec unconditioned stimulus (US) that consisted of 1 mA 60 Hz electric shock delivered to the grid floor. When the rat jumped on the ledge, the sequence was terminated. A 30 sec ledge rest period then ensued. If at the end of this time the animal still persisted in sitting on the ledge, it was automatically pushed off by an electromechanical moving wall. Random intertrial intervals were maintained with a mean of 30 sec and a range of 15–60 sec. After intraventricular injection, a 10 min period was allowed before animals were given 50 trials. The entire session lasted about 1 hr. Naive animals were given a total of 50 acquisition trials under the drug and discarded. Trained animals were given 50 trials per day for 5 consecutive days to achieve a 90% avoidance criterion and then used for the drug studies.

RESULTS

Comparative effects of acetylseco HC-3 and HC-3 on brain acetylcholine

In view of the fact that quaternary nitrogen derivatives do not readily penetrate the blood–brain barrier, both HC-3 and acetylseco HC-3 were given intraventricularly to young rats ½ hr prior to sacrifice. In Figure 2 the dose–effect relationships and per cent mortality are shown for groups of 6–10 rats per dose. Acetylseco HC-3 had a wider separation between

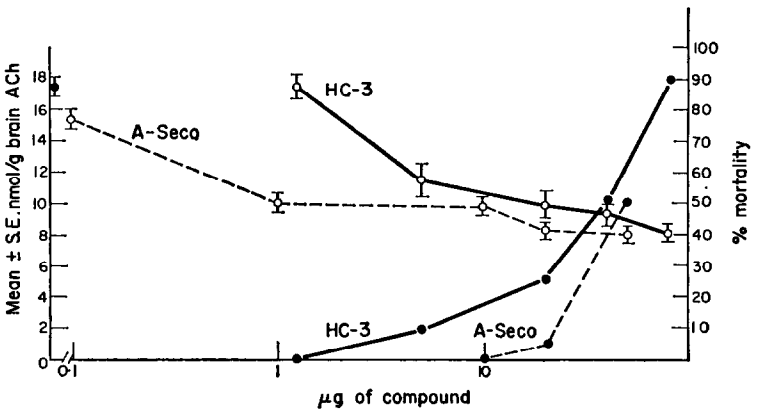


Fig. 2. Dose–effect relations of intraventricular acetylseco HC-3 and HC-3 on rat brain acetylcholine.

On the x axis is plotted the dose as base of the compound listed given intraventricularly. At 0 dose an equimolar amount of sodium bromide to 20 μg hemicholinium bromide was given. The mean $\pm$ S.E. for 6–12 rats is shown. Increasing doses of intraventricular acetylseco HC-3 (A-SECO) and HC-3 reduce total brain ACh and increase mortality. Note that acetylseco HC-3 is more potent in reducing brain ACh and is less toxic. All animals were killed $\frac{1}{2}$ hr after drug administration.

the dose that lowers ACh in brain and lethality than HC-3. Furthermore, it was much more effective in reducing ACh at lower doses than HC-3. HC-3 appeared to be a more toxic compound than acetylseco HC-3. With either compound in doses of 50 μg intraventricularly there was a 50% mortality. Animals that received HC-3 exhibited shaking, twitching, rigid posture, sometimes convulsions and respiratory arrest. These effects were especially obvious with larger doses. After acetylseco HC-3 rats did not exhibit any of the characteristic behavior induced by HC-3. In general, they were not very responsive to handling. Both drugs caused over a 50% loss of ACh with larger doses. After about 1 μg of acetylseco HC-3 and 20 μg HC-3, no further drop in ACh was produced in rats that survived up to $\frac{1}{2}$ hr after injection.

Time course of brain acetylcholine depletion following acetylseco HC-3 and HC-3

In young rats intraventricular acetylseco HC-3 and HC-3 in doses of 20 μg total produced similar rates of brain ACh depletion which was maximal 30–60 min after injection. Groups of 8–10 rats were injected and sacrificed at 5, 15, 30 and 60 min later. The effects of acetylseco HC-3 appeared to be somewhat more rapid in onset in lower doses intraventricularly but both drugs showed similar levels of depletion of brain ACh at 60 min.

Acetylseco HC-3 inhibition of choline acetyltransferase activity in vitro

Rat brain ChAc activity was determined *in vitro* at different concentrations of acetylseco HC-3 and HC-3 varying from 10^{-7} to 10^{-2} M. Choline was present at 10^{-2} M and acetyl CoA at $6{\cdot}4 \times 10^{-4}$ M. The results of one experiment are shown in Figure 3, plotted as percent of control ChAc activity. HC-3 had no effect on ChAc activity. In contrast, acetylseco HC-3 was an inhibitor with an I_{50} of 1×10^{-5} M under these substrate concentrations. When this experiment was repeated with different batches of acetylseco HC-3 and acetyl CoA, the I_{50} values varied from 5×10^{-6} M to 1×10^{-5} M. The fact that several batches of

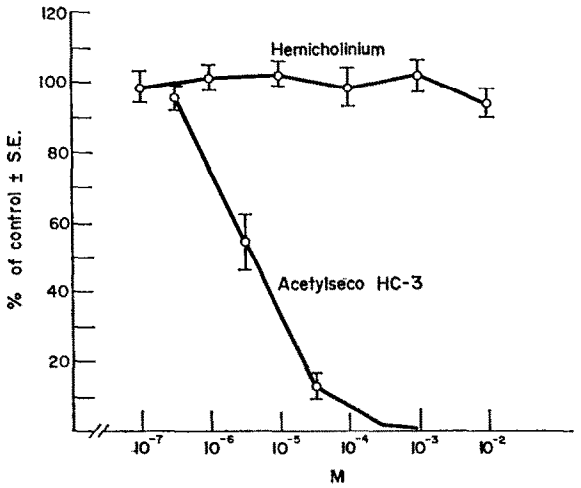


Fig. 3. Comparative effects of acetylseco HC-3 and HC-3 on choline acetyltransferase activity of rat brain homogenate.

On the x axis is given the molar concentration of the 2 compounds and on the y axis the per cent of control ChAc activity. The mean $\pm$S.E. of 4 separate determinations at each point is given. The substrate concentration for choline was 1×10^{-2} M and for acetyl CoA $6{\cdot}4 \times 10^{-4}$ M. Note that acetylseco HC-3 is an effective ChAc inhibitor with an I_{50} of 5×10^{-6} M at these substrate concentrations.

acetylseco HC-3 showed similar chemical analyses and caused similar *in vivo* decreases in brain ACh when given intraventricularly suggests that the change in I_{50} values is due to different batches of commercial acetyl CoA. This problem has been observed by others using commercial acetyl CoA preparations containing impurities (FONNUM, 1969; SCHUBERTH, 1971). Nevertheless, the results of the present experiments are clear in that acetylseco HC-3 is an inhibitor of ChAc activity but HC-3 is not.

The mode of inhibition of ChAc by acetylseco HC-3 seems complex. DEBALBIAN VERSTER *et al.* (1968) found that it was competitive with acetyl CoA and non-competitive with choline. Our own data with crude brain enzyme preparations and unpurified commercial acetyl CoA suggests it is mixed. In Figures 4 and 5 are the summarized data of individual experiments in which the reciprocal of varying concentrations of acetyl CoA and Ch are plotted against the reciprocal of enzyme activity. These particular Lineweaver–Burk plots were determined using 1×10^{-5} M acetylseco HC-3. Statistical estimates of the K_m and V_{max} were obtained using the computer analysis of WILKINSON (1961). In Figure 4 acetyl CoA concentrations varied from $3{\cdot}1 \times 10^{-3}$ M to $3{\cdot}1 \times 10^{-5}$ M. Choline concentration was 10^{-2} M. All other substrates in the incubation mixture were as described in the Methods. A V_{max} for acetyl CoA of $0{\cdot}28 \pm 0{\cdot}01$ μmol/g per min was obtained without acetylseco HC-3 and $0{\cdot}19 \pm 0{\cdot}02$ μmol/g per min after the inhibitor. The K_m without acetylseco HC-3 was $5{\cdot}1 \pm 0{\cdot}65 \times 10^{-4}$ M and $1{\cdot}4 \pm 0{\cdot}34 \times 10^{-3}$ M with 1×10^{-5} M acetylseco HC-3. These values depict a change caused by the presence of acetylseco HC-3 for both the V_{max} and the K_m of acetyl CoA. The results of an earlier experiment using different batches of drug and substrates also showed changes from normal in both V_{max} and K_m (see Table 1). The inhibition suggested by the use of a crude enzyme preparation and unpurified commercial acetyl CoA in the presence of acetylseco HC-3 is neither clearly competitive nor non-competitive but might be classified as mixed (WEBB, 1963).

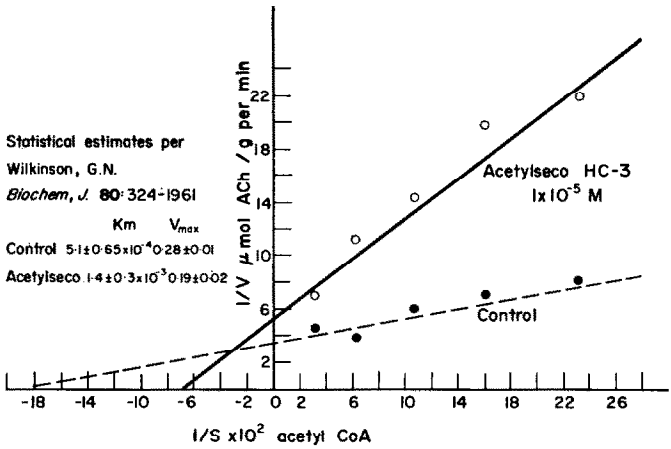


Fig. 4. Lineweaver–Burk plot of the inhibitory effects of acetylseco HC-3 on choline acetyltransferase activity of rat brain homogenate with varying acetyl CoA concentrations and constant choline.

Note that a mixed inhibition is observed. The K_m and V_{max} are given using the statistical estimate technique of WILKINSON (1961) for a range of $7{\cdot}5 \times 10^{-3}$–$4{\cdot}5 \times 10^{-6}$ M. A smaller range is shown for the individual experiment whose data are plotted on this graph.

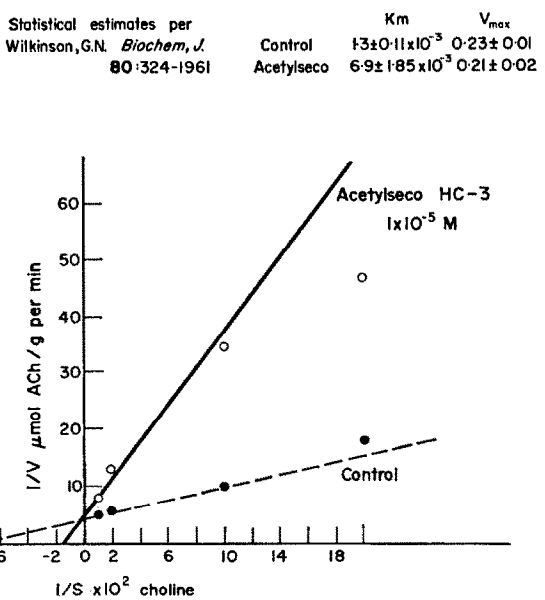


Fig. 5. Lineweaver–Burk plot of the inhibitory effect of acetylseco HC-3 on choline acetyltransferase activity of rat brain homogenate with varying concentrations of choline and constant acetyl CoA.

Note that a competitive inhibition is observed. The K_m and V_{max} are given using the statistical estimate techniques of WILKINSON (1961) for a range of 1×10^{-5} M. A smaller range is shown for the individual experiment whose data is plotted in this graph.

Table 1. Kinetic values for mean choline acetyltransferase activity ± S.E. of rat brain homogenate before and after acetylseco HC-3

Molar range	Choline control		Acetylseco HC-3 (5×10^{-6} M)	
	K_m (M) ± S.E.	V_{max} (μmol/g per min) ± S.E.	K_m (M) ± S.E.	V_{max} (μmol/g per min) ± S.E.
$1 M–10^{-7}$ M	$4{\cdot}02 \times 10^{-4} \pm 1{\cdot}12$	$0{\cdot}238 \pm 0{\cdot}009$	$1{\cdot}04 \times 10^{-4} \pm 0{\cdot}31$	$0{\cdot}236 \pm 0{\cdot}014$
10^{-3} M–10^{-5} M	$5{\cdot}4 \times 10^{-4} \pm 0{\cdot}74$	$0{\cdot}222 \pm 0{\cdot}008$	$6{\cdot}0 \times 10^{-4} \pm 1{\cdot}15$	$0{\cdot}182 \pm 0{\cdot}012$
10^{-2} M–5×10^{-5} M	$1{\cdot}1 \times 10^{-3} \pm 0{\cdot}19$	$0{\cdot}162 \pm 0{\cdot}009$	$1{\cdot}4 \times 10^{-3} \pm 0{\cdot}35$	$0{\cdot}119 \pm 0{\cdot}011$
10^{-2} M–10^{-5} M	$1{\cdot}3 \times 10^{-3} \pm 0{\cdot}11$	$0{\cdot}225 \pm 0{\cdot}006$	$6{\cdot}9 \times 10^{-3} \pm 1{\cdot}85$	$0{\cdot}206 \pm 0{\cdot}020$
	Acetyl CoA control		Acetylseco HC-3 (5×10^{-6} M)	
$7{\cdot}5 \times 10^{-3}$ M–$4{\cdot}5 \times 10^{-6}$ M	$3{\cdot}4 \times 10^{-4} \pm 0{\cdot}41$	$0{\cdot}388 \pm 0{\cdot}014$	$4{\cdot}3 \times 10^{-3} \pm 0{\cdot}5$	$0{\cdot}230 \pm 0{\cdot}008$
$3{\cdot}1 \times 10^{-3}$ M–$3{\cdot}1 \times 10^{-5}$ M	$5{\cdot}1 \times 10^{-4} \pm 0{\cdot}65$	$0{\cdot}284 \pm 0{\cdot}014$	$1{\cdot}43 \times 10^{-3} \pm 0{\cdot}34$	$0{\cdot}192 \pm 0{\cdot}017$

Lineweaver–Burk analysis of data obtained by varying Ch from 10^{-2} to 10^{-5} M is shown in Figure 5. Acetyl CoA was held constant at $6{\cdot}4 \times 10^{-4}$ M. A V_{max} for Ch of $0{\cdot}23 \pm 0{\cdot}01$ μmol/g per min without acetylseco HC-3 and $0{\cdot}21 \pm 0{\cdot}02$ μmol/g per min with acetylseco HC-3 was obtained. The K_m without acetylseco HC-3 was $1{\cdot}3 \pm 0{\cdot}11 \times 10^{-3}$ M and $6{\cdot}9 \pm 1{\cdot}9 \times 10^{-3}$ M with this inhibitor. It would appear that in this particular experiment, and at the molar ranges depicted, competitive inhibition is involved. Several other experiments performed over a period of one year with different batches of acetyl CoA and acetylseco HC-3 produced similar results, as shown in Table 1.

Effect of intraventricular acetylseco HC-3 and HC-3 on ^{14}C-choline incorporation into ^{14}C-acetylcholine in vivo

As described in the Methods, ^{14}C-Ch in a dose of 160 nmol and 1 μCi was given intraventricularly to unanaesthetized adult rats with indwelling polyethylene brain cannulae. A time interval of 10 min between injection and decapitation was chosen to minimize the effects of counting recycled ^{14}C-Ch and because our previous experiments indicated incorporation of at least 5% ^{14}C-Ch into ^{14}C-ACh at this time. The effects of acetylseco HC-3 and HC-3 on *in vivo* incorporation of ^{14}C-Ch into ^{14}C-ACh are shown in Figure 6. The values of at least 6 animals per drug are normalized as per cent change from control. Acetylseco HC-3 drastically reduced ^{14}C-Ch incorporation into ^{14}C-ACh to $15{\cdot}5 \pm 1{\cdot}1\%$ of control. HC-3 in equal amounts (20 μg) reduced ^{14}C-Ch incorporation to $48{\cdot}0 \pm 9{\cdot}6\%$. The mean ± S.E. extractable free pool of ^{14}C-Ch was $157{\cdot}5 \pm 15{\cdot}5\%$ after acetylseco HC-3 and only $118{\cdot}4 \pm 5{\cdot}7\%$ after HC-3. These differences are both statistically significant ($P < 0{\cdot}001$ for acetylseco HC-3 and $P < 0{\cdot}05$ for HC-3).

Comparative effects of acetylseco HC-3 and HC-3 on rat one way jump box avoidance and escape behavior

Groups of 8–12 rats were studied on acquisition and performance in a jump box following intraventricular equimolar NaBr and 20 μg intraventricular acetylseco HC-3 and HC-3. Both of these compounds reduced brain ACh to about 50% of control within ½ hr. Ten minutes after intraventricular injection rats were given 50 trials which lasted about 1 hr. Both naive and trained (90% avoidance criterion) animals were run. The data obtained are given in a bar graph format as mean per cent of total response ± S.E. for each series in

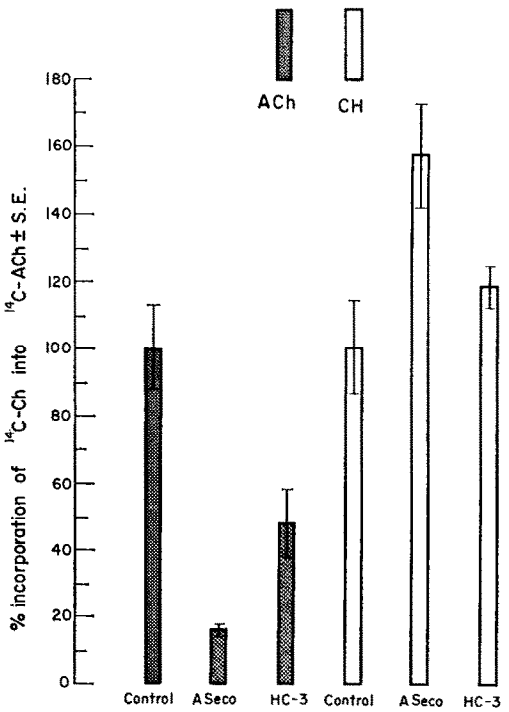


Fig. 6. Effects of acetylseco HC-3 and HC-3 on ^{14}C-choline incorporation into ^{14}C-acetylcholine in rat brain.

Both drugs (20 μg total) as well as ^{14}C-Ch (1 μCi total, Specific Radioactivity 61 mCi/mm) were given intraventricularly to groups of 6 adult rats with indwelling polyethylene cannulae. The drug and ^{14}C-Ch were given simultaneously and the animals sacrificed 10 min later. Control animals received 20 μl intraventricularly of 0·9% NaCl. The data are normalized so that control levels of ^{14}C-Ch incorporation in ACh represent 100%.

Figure 7. Both acetylseco HC-3 ($P < 0{\cdot}01$) and HC-3 ($P < 0{\cdot}001$) significantly reduced acquisition and increased escape responding of naive as well as trained animals. Naive animals were more affected than trained animals. HC-3 produced more behavioral toxicity than acetylseco HC-3 even though both drugs reduced total brain ACh to similar levels. Gross behavioral alterations such as tremors were also more obvious following HC-3.

DISCUSSION

Several classes of ChAc inhibitors are now known. All have some disadvantages as neuropharmacological tools. Sulphhydryl inhibitors such as iodoacetate and *p*-chloromercuribenzoate are relatively nonspecific (Reisberg, 1957; Mannervik and Sorbo, 1970). Cysteine is also a ChAc inhibitor but in excessive concentrations (Morris, Hebb and Bull, 1966). Several halogen derivatives of ACh are known to be uncompetitive inhibitors of ChAc in concentrations of 6×10^{-5} M (Persson, Larson, Schuberth and Sorbo, 1967; Morris and Grewaal, 1969; Henderson and Rama Sastry, 1972). Perhaps the best known inhibitors of this enzyme are the styrylpyridine analogues (Allen, Carlson and Cavallito, 1970; Cavallito *et al.*, 1969, 1970a,b; Smith, Cavallito and Foldes, 1967; White and Cavallito, 1970). To this list of ChAc inhibitors one can add acetylseco HC-3. The compound is clearly different from HC-3, although it does share other pharmacological actions

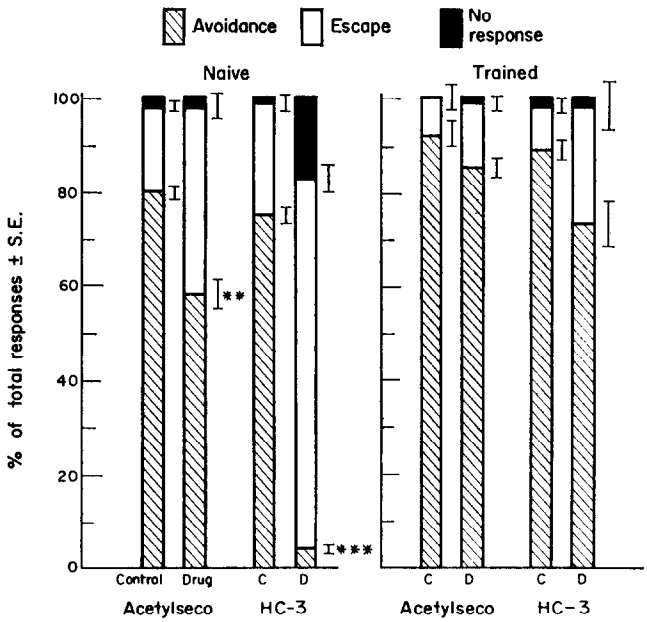


Fig. 7. Comparative effects of acetylseco HC-3 on rat one way avoidance and escape behavior. Both drugs were given intraventricularly in a total dose of 10 μg to adult rats with indwelling cannulae. Equimolar amounts of sodium bromide were given for acetylseco HC-3 and HC-3. The hatched bars represent avoidance, the open bars escape, and the solid black bars no response. After each treatment these add to 100% total responses. The height of each bar represents the mean and the small vertical lines $\pm$ S.E. for avoidance and escape data only. The data from naive animals is to the left and trained animals to the right. Note that both agents depressed acquisition of avoidance more than escape behavior in both naive and well trained animals but that HC-3 is more potent. All the animals were run 10 min after intraventricular injection for a total of 50 trials which lasted approximately 1 hr. Note that both drugs affected naive more than trained animals. Each bar graph represents the mean $\pm$ S.E. of 8–12 animals. A group comparison *t*-test of control and drug treated groups was done. Significant differences of *P* for avoidance behavior are shown as ** $< 0{\cdot}01$ and *** $< 0{\cdot}001$.

of HC-3, including reduced toxicity after Ch (MAGGIO, 1968; MAGGIO and HAARSTAD, unpublished observations). The fact that acetylseco HC-3 is a bischoline ester makes it susceptible to hydrolysis, although strangely it has not been reported to be a substrate for cholinesterase *in vitro*.

Studies of the action of intraventricular HC-3 and acetylseco HC-3 on total rat brain ACh indicate that both reduce ACh, although with different doses, times and lethality. Because of these differences, a study of the *in vitro* effects of these compounds on rat brain ChAc was deemed important. HC-3 was shown to have no effect while acetylseco HC-3 was found to be a potent inhibitor (Fig. 3), an indication that the two compounds were both reducing brain ACh through different mechanisms. This does not rule out, however, the possibility that acetylseco HC-3 also affects the transport of Ch in a manner similar to HC-3. Like HC-3, acetylseco HC-3 is found to markedly alter normal liver metabolism producing fatty liver (MAGGIO and HAARSTAD, unpublished observations). The fact that Ch reverses many of the pharmacological actions of acetylseco HC-3 is further suggestion of an action on Ch transport.

Kinetic studies using Lineweaver–Burk analysis do indicate that acetylseco HC-3 is a competitive inhibitor of Ch. Acetylseco HC-3 also has many similarities with ACh. MAGGIO

and HAARSTAD (unpublished observations) found acetylseco HC-3 to be a potent parasympathomimetic additive with ACh. HC-3 did not exhibit these properties. Ileum contractions elicited by acetylseco HC-3 were blocked by atropine but not hexamethonium. Studies of base-catalyzed hydrolysis indicate that the ACh moiety of acetylseco HC-3 is similar in chemical reactivity to ACh. If acetylseco HC-3 has similarities to ACh, it could be acting like ACh through substrate inhibition of ChAc. KAITA and GOLDBERG (1969) have shown that increasing amounts of ACh produce competitive inhibition with Ch. If acetylseco HC-3 is occupying the choline site on the enzyme (as ACh would), there would be no acetylation of Ch. There would be no competition for the enzyme site normally occupied by acetyl CoA. Our enzyme kinetics, however, indicate a mixed type of interaction with acetyl CoA. It might be that the greater size of the acetylseco HC-3 molecule is creating a physical hindrance to the attachment of acetyl CoA to the enzyme site.

Of special importance are further studies using purified ChAc to better define the kinetics of inhibition by acetylseco HC-3. The studies reported in this paper should be regarded as preliminary in view of the use of commercially available ^{14}C-acetyl CoA and rat brain homogenate as the enzyme source. In spite of these shortcomings, the data indicate that acetylseco HC-3 may become an important neuropharmacological tool. Results of intraventricular injections of acetylseco HC-3 or HC-3 simultaneously with pulse doses of ^{14}C-Ch further indicate that acetylseco HC-3 has a more potent effect than HC-3 on incorporation of Ch into ACh. Again, this does not eliminate the possibility that acetylseco HC-3 affects Ch transport as well as inhibition of ChAc. Its greater potency than HC-3 for reduction of ACh could be due to the combined effects of the two actions. DIAMOND and KENNEDY (1969) have shown that HC-3 inhibits incorporation of choline into phosphorylcholine. The possibility that acetylseco HC-3 may also do the same must also be studied.

The behavioral effects of intraventricular acetylseco HC-3 as well as HC-3 indicate that these agents are useful in altering brain function in animals. Unexpectedly HC-3 produced greater behavioral toxicity than acetylseco HC-3. Furthermore, tremors were also more obvious after HC-3, suggesting it has further differences in action from acetylseco HC-3.

Of special interest is the fact that 4-(1-naphthylvinyl) pyridine hydrochloride (NVP), one of the potent styrylpyridine derivatives of CAVALLITO *et al.* (1969, 1970a, b), had negligible behavioral effects in rats unless massive doses were given i.p. (GOLDBERG, SLEDGE, ROBICHAND and DUBINSKY, 1972). Of course, this may have been because NVP does not readily penetrate the blood–brain barrier. In the present study acetylseco HC-3 was given intraventricularly to avoid its obvious peripheral effects when given parenterally. Further studies comparing intraventricular NVP and acetylseco HC-3 on various animal behaviors and possible antagonism by Ch would be of considerable interest.

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