Influence of Hemicholinium No. 3 on Mammalian Tissue Levels of Acetylcholine.* (30370)

LARRY G. HART AND J. P. LONG
Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City

Hemicholinium No. 3 (HC-3), the *bis*-hemiacetal form of a,a'-dimethyl ethanolamino-4,4′-biacetophenone was synthesized originally by Long and Schueler(1) as a member of a series of biphenyl derivatives that were potent inhibitors of cholinesterase. HC-3 was essentially devoid of this type of activity, but was later shown to interfere with cholinergic transmission(2). This compound inhibits acetylcholine (ACh) synthesis in the cat superior cervical ganglion preparation(3). Further studies(4,5,6) resulted in the hypothesis that HC-3 competes with the transport of choline from the extracellular fluid to sites of acetylation. Administration of choline antagonizes the inhibitory action of HC-3.

Since ACh is virtually depleted from the perfusate of the cat superior cervical ganglion (6) this could be suggestive of either inhibition of synthesis or an anti-release mechanism for HC-3. Their evidence does not exclude the possibility of anti-release action for HC-3 at other cholinergic sites. Lewartowski and Bielecki(7) using rabbit atria *in situ* in the presence of HC-3, established that stimulation of vagal nerves until complete disappearance of responses in animals resulted in a reduction of atrial ACh to 33% of control values. Hemicholinium or vagal stimulation applied separately did not affect the atrial ACh content.

The purpose of this project was to study resting levels of ACh in various cat tissues after single and repeated injections of HC-3. Further, an attempt was made to compare these levels with the levels of ACh after both HC-3 and *in situ* nerve stimulation.

Materials and methods. Cats of either sex, weighing 2-3 kg, were used for the *in situ* studies.

In situ *experiments:* In acute experiments, cats were anesthetized with an intrathoracic injection of sodium pentobarbital, 30 mg/kg. Both vagi and the left sciatic nerve were stimulated electrically. The vagi were stimulated for 10 seconds every 2 minutes. The parameters used were a frequency of 20/sec, a biphasic pulse of 5 milliseconds duration and 15 volts. The sciatic nerve was stimulated with supramaximal voltage, a single shock each second of 5 milliseconds duration. One-half of the animals received HC-3, 1 mg/kg, by the intravenous route. Forty minutes after the beginning of stimulation, the ACh contents of the auricles, intestines and gastrocnemius muscles were bioassayed by the procedure described below. Also, resting levels of ACh were determined in iris and ciliary bodies after topical administration of a 1% solution of HC-3 to both eyes. Administration of HC-3 was repeated in 2 to 3 hours, allowing maintenance of maximal mydriasis up to the time of sacrifice, or 5 hours after the initial treatment.

For the more chronic experiments, another series of cats were anesthetized and the trachea cannulated and attached to a Palmer pump to respire the animals artificially. HC-3, 1 mg/kg, was given intramuscularly to half of the cats. This dose was given every 6 hours and the animals maintained maximal mydriasis (our end point) for the duration of the experiment, 24 hours after initial administration of drug.

Acetylcholine extraction and estimation. The various tissues (irises and ciliary bodies, gastrocnemius muscles, auricles and small intestines) were excised and immediately frozen in liquid nitrogen. The trichloroacetic acid extraction as described by MacIntosh and Perry(8) was used with minor modifications, and bioassay of the subsequent extracts was done using the eviscerated cat blood pressure preparation, also as described by these authors. Injections of standards and unknowns were made into the inferior vena cavae just below the junction with the renal vein, rather than into the jugular or femoral

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TABLE I. ACh Levels of Cat Tissues After Acute Administration of Hemicholinium (HC-3).*

Tissue	Nerve stimulation†		Non-stimulated‡	
	HC-3, 1 mg/kg	Control	HC-3, 1 mg/kg	Control
Iris and ciliary body			1.1 ± .37 (8)	1.5 ± .24 (10)‖
Skeletal muscle	.6 ± .26	.6 ± .32	1.2 ± .52	.9 ± .48
Intestine	4.64 ± .70 (5)	4.7 ± .45 (5)	2.9 ±2.96 (3)	3.2 ±1.61 (3)
Auricles	.86 ± .31 (5)	2.2 ± .56 (5)§	1.1 ± .19 (3)	1.6 ± .79 (3)

* Values in Table present μg ACh/g tissue wet wt ± standard deviation. Numbers in parentheses indicate animals per group.

† Stimulation of both vagi or sciatic nerve 40 min after HC-3, 1 mg/kg I.V.

‡ Tissues were extracted 3 to 5 hr after drug administration I.V. Iris and ciliary bodies were assayed after topical administration of 1% HC-3 solution to both eyes.

§ $P < .001$.

‖ $P = .05$-$.02$. For all other assays $P > .05$.

TABLE II. ACh Levels of Cat Tissues After "Chronic" Administration of Hemicholinium (HC-3).*

Tissue†	HC-3	Control	P
Iris and ciliary body	3.2 ± 1.83 (13)	4.0 ± 1.55 (10)	.3–.2
Skeletal muscle	2.4 ± 1.34 (5)	1.8 ± .89 (4)	.5–.4
Intestine	12.7 ± 8.84 (7)	12.5 ± 6.05 (5)	>.5
Auricles	1.4 ± .81 (7)	1.9 ± 1.56 (5)	>.5

* Values in Table represent μg ACh/g tissue wet wt ± standard deviation. Numbers in parentheses indicate animals per group.

† Animals were anesthetized and artificially respired. HC-3, 1 mg/kg, was given I.M. at beginning and every 6 hr until sacrificed 24 hr after initial administration of HC-3.

vein as in their preparation. At least 3 different volumes of each extract were given, bracketed at appropriate intervals by 4 doses of standard.

The Student "t" test was utilized to analyze statistical significance of any differences between the means of experimentals and controls. A P value equal to or less than .05 was regarded as significant.

Results. In situ *experiments.* In the acute experiments, HC-3 appeared to have little effect on the levels of ACh in the various tissues studied with one exception, that being the levels in the auricles after stimulation of both vagi (Table I). Here the effects of HC-3 seemed to prevent the increases of ACh content usually observed after nerve stimulation. In the control auricles and in the other tissues, except skeletal muscle, the mean values for the stimulated tissues were higher than the resting values, although due to considerable variation these differences were not statistically significant. In the skeletal muscle, nerve stimulation produced a significant reduction in amount of extractable acetylcholine ($P = 0.02$-0.05). In the irises and ciliary bodies, there was a significantly greater amount of ACh in the controls compared to those treated topically with HC-3, although levels in the latter were not severely reduced. Though quite small, this reduction in ACh may be responsible at this site for inhibition of cholinergic transmission.

When HC-3 was administered as a dose of 1 mg/kg I.M. every 6 hours for a 24-hour period and in the absence of nerve stimulation, there were no significant differences in ACh levels between treated and control in any of the tissues studied (Table II). With the notable exception of the auricles, the mean tissue levels were considerably higher than in those tissues from the acutely treated animals. It is not readily apparent whether any meaning can be assigned to these differences.

Discussion. It has been well documented that nerve stimulation is required for hemicholinium No. 3 (HC-3) to effect a blockade of cholinergic transmission *in situ* and further there appears to be a direct relationship between degree of depression and frequency of stimulation(9-12). Our results in non-stimulated tissues of the cat were consistent with the above observations in that one would

expect much less of an alteration in ACh levels by HC-3 if electrical stimulation were not applied concurrently.

The HC-3 administration in the eye for 24 hours produced a mydriatic and cycloplegic effect, but without any significant alteration of total ACh levels in the affected structures. These results were consistent with the report that the mydriasis is antagonized readily by physostigmine and DFP, thus indicating that ACh was still present in functional amounts(13). Denervation of 3-4 weeks duration of the ciliary ganglion results in ACh depletion of the iris and ciliary body. Cholinesterase inhibitors are ineffective as miotic agents (Long and Armaly, unpublished results).

The auricles appear to be unique of the tissues investigated. Only in the auricles was there a significant reduction of ACh levels after administration of HC-3, and then only if they were stimulated. The data in the cat auricles *in situ* parallel that obtained by Lewartowski and Bielecki(7) using rabbit auricles. These workers feel that there are three fractions of ACh in the auricles, a very small nervous fraction and a larger, extraneural fraction which together constitute about 60% of the total and are releasable by HC-3 and nerve stimulation, plus a second extraneural fraction not releasable. The role of these fractions is uncertain. However, no significant changes were observed in the basic auricular activity after depletion of the two releasable fractions.

Whether or not these postulated fractions are present in the other tissues has yet to be resolved, and furthermore it is not known how much of the ACh content of these tissues is extraneural. Morley and Schachter(14) have found rather large amounts of ACh in non-nervous tissues of some lepidoptera and speculated that functions of ACh other than that of chemical mediation of synaptic transmission remain to be discovered. Hayes and Riker(15) demonstrated that the amounts of ACh liberated by innervated and chronically denervated paired hemidiaphragms did not differ, whether the muscles were at rest or electrically stimulated. It has been demonstrated that HC-3 is capable of reducing markedly the amount of ACh liberated using the isolated phrenic nerve-diaphragm preparation of the rat(16). Since we were unable to detect any significant differences in tissue levels of ACh after HC-3, with the two exceptions mentioned, a possible anti-release mechanism of HC-3 should be considered. If inhibitory effects by HC-3 on ACh synthesis were the mechanism for producing transmission failure in all preparations, one would expect lower levels of the neurohormone in the non-cardiac tissues after repeated administration of HC-3 and certainly after nerve stimulation. Defective synthesis would not explain the lack of differences, unless most of the ACh were extraneural and not affected by HC-3. Perhaps in those tissues where HC-3 was ineffective in lowering ACh levels, the neural ACh is small in relation to the possible extraneural sources.

Summary. Hemicholinium No. 3 (HC-3) was administered acutely to cats, either as a 1% solution topically to the eyes or an i.v. injection of 1 mg/kg, and tissue levels of acetylcholine (ACh) were determined. The effects of nerve stimulation on ACh levels were also studied in these tissues, which included auricles, intestines and gastrocnemius muscles. Levels of ACh were significantly greater in control resting irises and ciliary bodies than in those treated with HC-3. The content of the auricles were also greater in the controls but only if nerve stimulation had been previously applied. Otherwise there were no differences between tissues from HC-3 treated and control animals. Another group of cats received a more chronic treatment with HC-3, 1 mg/kg repeated i.m. every 6 hours for a 24-hour period. Analysis of the same tissues for resting ACh content revealed no differences between treated and control animals. The results were discussed with regard to HC-3 exerting a possible anti-release mechanism. The possibility was also considered that much of the ACh might be of extraneural origin and not affected by HC-3.

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Failure of Urecholine to Potentiate the Pancreatic Response to Exogenous Stimuli.† (30371)

R. M. PRESHAW,* K. ADASHEK, A. R. COOKE AND M. I. GROSSMAN

Research, Veterans Administration Center, Los Angeles, and Department of Medicine, UCLA Center for the Health Sciences, Los Angeles, Calif.

Parasympathomimetic agents have been shown to potentiate the action of the hormone of the pyloric gland area, gastrin, on gastric secretion(1). It has frequently been suggested that potentiation between cholinergic effects and other stimuli might occur in the regulation of pancreatic secretion(2).

In this study we have attempted to determine whether there is potentiation by a stable choline ester, Urecholine, of pancreatic secretion induced by the normal hormonal stimulants of the pancreas.

Methods. Four mongrel dogs weighing between 14 and 21 kg were used. A chronic pancreatic fistula was created surgically in each animal by a method we have described (3). At a second operation, a Thomas cannula(4) was inserted into the most dependent portion of the stomach as a gastric fistula. At least 4 weeks were allowed for recovery from this procedure before experiments were undertaken.

After fasting for 18 hours, the pancreatic and gastric fistulas were opened and secretions collected for at least 30 minutes to confirm basal levels. A fine plastic catheter was introduced intravenously and an infusion of sterile 0.15 M NaCl maintained throughout each experiment by a Harvard peristaltic pump at a rate of 30 ml per hour. The stimulant under test was dissolved in the saline to give appropriate dose levels.

Gastrin was extracted from the pyloric gland area mucosa of hog stomachs by the method of Grossman and Gillespie(5). One large batch of gastrin was prepared of sufficient size for both parts of this study to ensure comparable dose-response curves. Similarly the secretin and Cecekin used came from the same batch numbers as obtained from the manufacturer (Vitrum, Stockholm).

Dose-response relations were studied for pancreatic and gastric secretion evoked by intravenous infusion of secretin, Cecekin, and gastrin. Each dose level was maintained for 60 minutes in the case of secretin and Cecekin, and for 75 minutes for gastrin. Secretions were collected every 15 minutes, and mean values of the last two 15-minute collection periods at any one dose level used to construct the dose-response curves shown in the Figures.

A dose-response curve was also determined

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