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PERIPHERAL TOXICITY OF HEMICHOLINIUM-3 IN MICE

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1 The site (i.e. peripheral or central) of the toxicity produced by hemicholinium-3 in mice was investigated.

2 Hemicholinium-3 was measured fluorometrically and acetylcholine was determined by gas chromatography after intraventricular or intraperitoneal administration of hemicholinium-3.

3 Hemicholinium-3 was not detected in the brain nor were acetylcholine levels decreased in the brain after systemic administration.

4 The dose-response curve following intraventricular administration demonstrated that hemicholinium-3 was not as lethal after central administration as it was after peripheral administration.

5 Approximately 24% of a 75 μg intraventricular dose of hemicholinium-3 was found in the periphery at death.

6 These results suggest that hemicholinium-3 manifests its toxicity primarily in the periphery.

Introduction

Hemicholinium-3 [2,2-(4,4-biphenylene)-bis-(2-hydroxy-4-dimethylmorpholinium)-bromide] (HC-3) acts to inhibit acetylcholine synthesis *in vitro* by blocking the uptake of choline into the cholinergic neurone (MacIntosh, Birks & Sastry, 1956; Gardiner, 1961; Guynet, Rossier, Beaujouan & Glowinski, 1973). An inhibition of high affinity choline uptake at the cholinergic nerve terminal also appears to provide a satisfactory explanation of its effects *in vivo* (Freeman, Macri, Choi, & Jenden, 1979). However, HC-3 also produces other effects besides inhibition of choline re-uptake. It exhibits antimuscarinic activity (Bertolina, Creggia & Ferrari, 1967; Madden & Mitchelson, 1975), and postjunctional blockade at the skeletal muscle motor endplate (Schueler, 1955; Marshall, 1969). Furthermore, HC-3 may be acetylated and subsequently preferentially released as an inactive 'false' transmitter (Collier & MacIntosh, 1969; Hemsworth, 1971). Thus, although the actions of HC-3 cannot be interpreted solely in terms of reduced synthesis of acetylcholine, virtually all of the actions appear to contribute to a reduced interaction at the receptor (Elmquist & Quastel, 1965).

In spite of the well-known inability of quaternary ammonium compounds to penetrate the blood-brain barrier, the site of HC-3 toxicity continues to be controversial. Schueler, Longo & Bovet (1954), Domer & Schueler (1960), Longo (1958; 1959) and Dren & Domino (1968) presented evidence that HC-3 produces its toxicity in the periphery. Schueler (1955), Kase & Borison (1958), Metz (1960), Quastel & Curtis (1965) and Clement (1978) were of the opinion that the toxicity produced by HC-3 after systemic administration was mainly due to a depression of the respiratory regulatory mechanism in the central nervous system. The purpose of the present study was to determine whether the fatal respiratory paralysis produced by HC-3 is centrally or peripherally mediated.

Methods

Male HA/ICR mice weighing between 25 and 35 g were used in this study. Mice were supplied by Harlan Sprague Dawley Industries, Indianapolis, Indiana. Hemicholinium-3 was obtained from Aldrich Chemical Co., and neostigmine bromide, (+)-tubocurarine Cl, and atropine methyl bromide were obtained from Sigma Chemical Co.

Procedure for intracerebroventricular cannulation

The procedures of Haley & McCormick (1957), De Balbian-Verster, Robinson, Hengeveld & Bush (1971) and Shaw (1974) were modified to allow intracerebroventricular injection in conscious mice. Briefly, the animal was lightly anaesthetized with ether, grasped behind the head and the skin pulled taut. An incision the length of the sagittal structure was made and the cannula hole (0.024 inch) was

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drilled 1 mm lateral to the sagittal suture and 2 mm rostral to the coronal suture. The cannula was constructed according to a modification of the procedure of Robinson, Hengeveld & De Balbian-Verster (1969). The cannula was constructed from PE 10 tubing (Clay Adams) with a thick-walled bulb 3 mm from the bevelled end of the cannula. An anchoring screw for the cranioplastic cement was placed in the opposite side of the cranium. The animal was allowed 12 h to recover from the surgery.

Injections were performed in conscious mice with a 10 μl Hamilton syringe. Injection volume varied between 1.2 and 3.2 μl. The larger volume was used only when necessary at higher concentrations (i.e. 75 μg) of HC-3. The injection volume included the drug solution followed by 0.2 μl air and 0.5 μl saline. Following injection the cannula was sealed. Cannula placement was verified histologically by injecting toluidine blue dye intracerebroventricularly and sectioning the brain. Identical volumes of saline injected into controls did not produce measurable changes in ACh or behaviour. Current convention is utilized in expressing the intracerebroventricular dose in μg and the intraperitoneal dose in μg/kg. Conversions from amount injected to a per kg dosage are based on an average mouse weight of 30 g.

Measurement of acetylcholine, choline (Ch) and hemicholinium-3 in mouse tissue

The gas chromatographic assay of Kosh, Smith, Sowell & Freeman (1979) was used to determine brain levels of acetylcholine (ACh) and choline. Following decapitation, mouse brains were homogenized in 2.5 ml of 15% 1N formic acid-85% acetone.

The fluorescent assay of Freeman, Choi & Jenden (1975) was used to determine brain levels of HC-3. The method was modified as described below for the whole body assay. The body less the spinal cord and brain was homogenized in a Waring blender in 90 ml of 15% 1N formic acid-85% acetone for 1.5 min. The homogenate was then centrifuged at 26,000 *g* for 20 min. One fifth of the supernatant was carried through the remainder of the assay. Diethyl ether (40 ml) was added and the sample mixed by hand for 1 min. After 5 min, the ether phase was aspirated, and a second ether wash of 10 ml was then added. After aspiration of the ether phase, residual ether and acetone were removed by a stream of dry nitrogen. The sample was placed on ice and cold tetraethyl ammonium chloride (10 mM, 0.2 ml) added followed by 0.6 ml cold saturated (2%) ammonium reineckate salts. The sample was vortexed and placed in an ice bath for 30 min. The remainder of the assay was identical to Freeman *et al.*, (1975). The sensitivity of the assay was 0.1 μg HC-3. Recovery through the assay varied between 75 and 85%. All data were corrected for percentage recovery. A Perkin Elmer type MPF 44A spectrofluorometer was used with an activation wave-length of 260 nm and an emission wavelength of 310 nm with slit widths of 5 nm.

Toxicity measurements

HC-3 (150 μg/kg i.p.) was injected simultaneously with several compounds and the time to death noted. A dose of 150 μg/kg i.p. was adopted from the study of Clement (1978) since it was confirmed in the present study to produce 100% lethality. A dose of (+)-tubocurarine (300 μg/kg, i.p.) was selected since it produced nonlethal skeletal muscle relaxation, as tested on the rotorod, (15 rev/min, 1 in. diam). Neostigmine (0.33 mg/kg) caused salivation, lacrimation, urination and defaecation (SLUD syndrome, Squire, Glick & Golgfar, 1971) with lethality. A dose of 1 mg/kg atropine caused muscarinic blockade without lethality (Barnes & Eltherington, 1966).

Results

Effect of intraventricular (i.c.v.) doses of hemicholinium-3 on the time to death and percentage mortality in mice

Several doses of HC-3 were injected i.c.v. in an attempt to identify the lowest dose which would cause 100% mortality (Figure 1). There was a linear

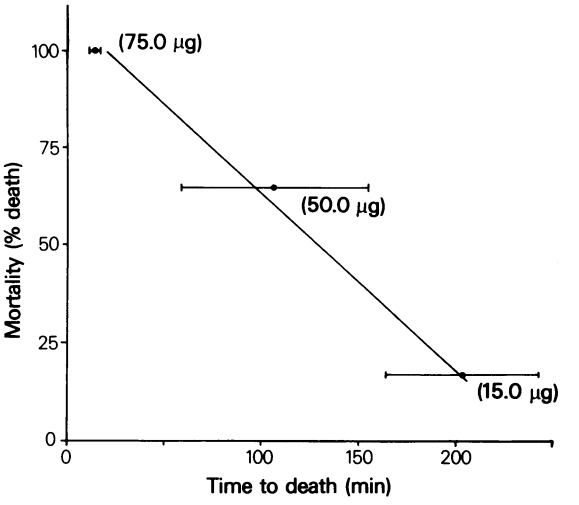

Figure 1 Effect of intracerebroventricular (i.c.v.) doses of hemicholinium-3 (HC-3) on the time to death and percentage mortality in mice. Dose of HC-3 (i.c.v.) is indicated in parentheses; 100% mortality was observed with 75 μg or 150 μg (not shown). A 5.0 μg i.c.v. dose (not shown) was nonlethal. Horizontal bars indicate s.e.mean; $n = 6$.

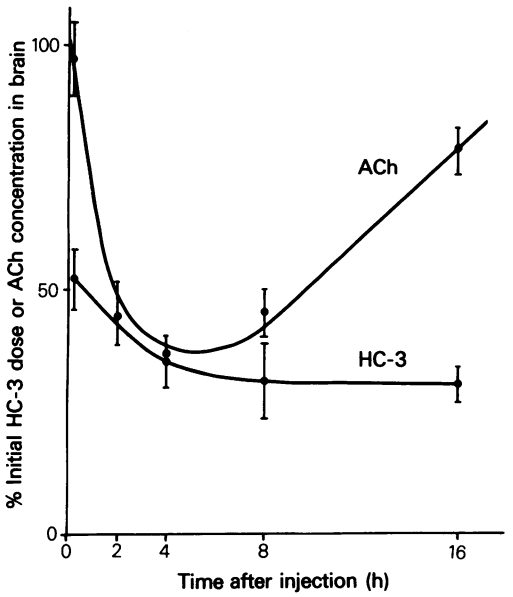

Figure 2 Time course of acetylcholine (ACh) and hemicholinium-3 (HC-3) concentration in mouse brain after intraventricular (i.c.v.) injection of 5 μg HC-3. Values are expressed as percentage initial level; vertical lines show s.e.mean. Control levels of ACh and choline were 9.0 ± 1.3 nmol/g and 39.5 ± 1.3 nmol/g respectively. Initial HC-3 level was the amount injected (5 μg). First measurement was made 10 min following the i.c.v. injection. Choline levels did not change significantly compared to controls.

relationship between time to death and % mortality. Both 75 μg and 150 μg HC-3 (not shown) caused 100% mortality within 15 min while 83% of the animals survived for more than 3 h following a 15 μg i.c.v. dose. Both the 50 μg and 15 μg i.c.v. doses caused a wide variability in the time to death while 5.0 μg i.c.v. HC-3 did not cause any deaths. The LD_{50} for i.c.v. HC-3 in the mouse is approximately 35 μg. Behaviourally, death was preceded by respiratory difficulties.

Time course of acetylcholine and hemicholinium-3 concentration following intraventricular injection of HC-3 in mice

The purpose of this experiment was to determine the time course of ACh and HC-3 levels after a non-lethal 5 μg i.c.v. dose of HC-3 (Figure 2). ACh levels decreased to 39% of control values at 4 h and returned to 80% of control values at 16 h. Only 51% of the amount of HC-3 injected was found in the brain after 10 min, but 29% was still in the brain after 16 h.

Effect of hemicholinium-3 (i.p. or i.c.v.) on the time to death and on the concentration of acetylcholine, choline and HC-3 in mice

The purpose of this experiment was to examine whether there is a relationship between the level of ACh, HC-3 and the time to death of a lethal dose of HC-3. A lethal i.p. dose of HC-3 (150 μg/kg, i.p.) did not produce detectable levels in the brain and did not significantly change the ACh concentration (Table 1). A 75 μg i.c.v. dose caused a significant reduction in brain ACh at death (15.4 min) as well as a significant increase in brain choline. Only 50.6 μg of HC-3 was found in the brain at death, and 18.2 μg was found in the body. When 18.2 μg was converted to a per kg dose (622 μg/kg, methods) and injected i.p., the time to death was not significantly different from the time to death caused by 75 μg i.c.v. The distribution of HC-3 after an i.c.v. or i.p. dose is summarized in Figure 3.

Table 1 Effect of dose† of hemicholinium-3 (HC-3) on the time to death and on the concentration of acetylcholine (ACh), choline (Ch) and HC-3

		Concentration			
HC-3 dose	*Time to death (min)*	*ACh (nmol/g) Brain*	*Ch (nmol/g) Brain*	*HC-3 (μg) Brain*	*HC-3 (μg) Body*
150 μg/kg (i.p.)	14.8 ± 1.9	11.4 ± 0.8	48.3 ± 2.1	ND	(−)
75 μg (i.c.v.)	15.4 ± 2.4	6.0 ± 0.4*	75.8 ± 9.5**	50.6 ± 2.7	18.2 ± 2+°
622 μg/kg (i.p.)+°	11.4 ± 0.6	(−)	(−)	(−)	(−)
Control	(−)	9.0 ± 0.3	39.5 ± 1.3	(−)	(−)

†Dose administered intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.)
Values are expressed as mean ± s.e.mean; (−) indicates not measured; ND = non-detectable. $n = 6–10$.
*$P < 0.02$; **$P < 0.01$.
+°18.2 μg represents 622 μg/kg which was reinjected i.p.

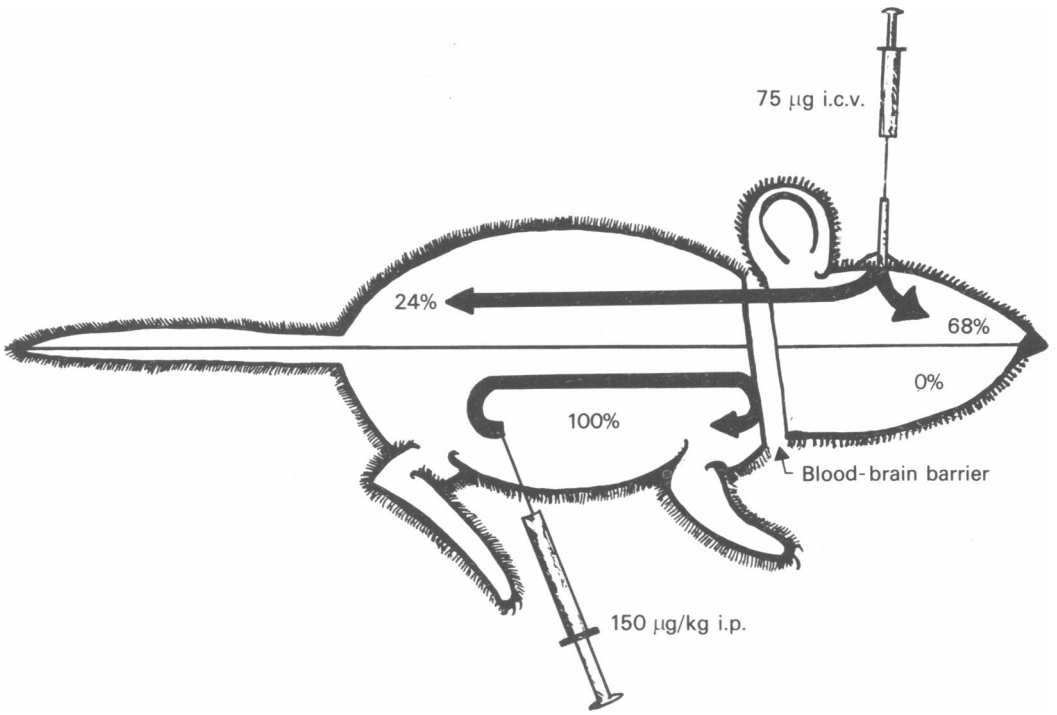

Figure 3 The distribution of hemicholinium-3 (HC-3) after intraventricular (i.c.v.) or intraperitoneal (i.p.) administration.

Effect of drugs on the time to death caused by hemicholinium-3

The purpose of this experiment was to alter the time to death of peripherally administered HC-3 with drugs which act at peripheral nerve terminal sites (Table 2). Simultaneous intraperitoneal administration of neostigmine or (+)-tubocurarine with hemicholinium-3 produced an increase (25 min) and a decrease (7 min) respectively in the time to death compared to the time produced by HC-3 alone (15 min). Atropine methyl bromide produced no significant change in time to death compared to control.

Discussion

HC-3 is known to inhibit ACh synthesis (Yamamura & Snyder, 1973) and to result in a depletion of ACh in brain (Domino, Cassano & Placidi, 1974; Freeman *et al.*, 1975). However, the present study does not support a relationship between toxicity and ACh depletion. A non-lethal 5 μg intraventricular dose produced a 61% depletion of ACh compared to only a 33% depletion after a lethal 75 μg i.c.v. dose (Figure 2, Table 1). A lack of correlation between ACh depletion and toxicity has also been observed in the rat (Freeman *et al.*, 1979). Hemicholinium-3 has also

Table 2 Effect of drugs on the time to death caused by hemicholinium-3 (HC-3, 150 μg/kg, i.p.)

Drug	*Time to death* (min)
HC-3 + (+)-tubocurarine (300 μg/kg, i.p.)	6.5 ± 0.8*
HC-3 + neostigmine (330 μg/kg, i.p.)	25.5 ± 3.0**
HC-3 + atropine methyl bromide (1 mg/kg, i.p.)	9.5 ± 0.7
HC-3 + saline	14.8 ± 1.9

†Nonlethal doses of (+)-tubocurarine, neostigmine or atropine methylbromide were administered simultaneously with HC-3.
Values are expressed as mean ± s.e.mean. $n = 6$.
*$P < 0.01$; **$P < 0.05$.

been reported to cause an increase in brain choline occasionally (Robinson, 1970; Domino, Mohrman, Wilson & Haarstad, 1973; Freeman *et al.*, 1979; Jope & Jenden, 1979). The explanation for the increase in brain choline observed in this and previous studies is unknown.

The results of this study indicate that HC-3 acts at a peripheral site in mice to cause toxicity. For example, HC-3 was not found in the brain at death after intraperitoneal administration. Since the sensitivity of the assay is 0.1 μg, and more than 5 μg must be given intraventricularly (Figure 1) before toxicity is observed, the data do not support a central site of toxicity. The present study confirms the findings of Domer & Schueler (1960) and others that HC-3 does not cross the blood-brain-barrier in significant amounts. Freeman *et al.*, (1979) had previously shown that i.c.v. injection of 0.1 μg HC-3 caused a 30% reduction in ACh in the brains of rats. A decrease in ACh levels would have indicated the presence of small amounts of HC-3 (Giarman & Pepeu, 1962) in the mouse brain after systemic administration. No decrease in ACh levels was observed in the present study substantiating the evidence that HC-3 does not readily cross the blood-brain-barrier.

Further evidence suggesting a peripheral site of action was provided by the dose-mortality curve (Figure 1). The intraventricular dose (75 μg) of HC-3 was 16.7 times the intraperitoneal dose (i.e. 4.5 μg or 150 μg/kg i.p., Clement, 1978) which caused 100% lethality. These results suggested that hemicholinium-3 is more potent in producing toxicity after peripheral administration. An explanation for the reduced toxicity after central administration was found by measuring the brain levels of HC-3 (Figure 2). A large percentage of the hemicholinium-3 dose was not recovered in the brain after 10 min. Shaw (1974) demonstrated that leakage of iophendylate into the periphery had occurred after intraventricular administration in mice. Therefore, leakage into the periphery after intraventricular administration of HC-3 was a possible explanation for the loss. Assay of the body for HC-3 after a lethal intraventricular dose of 75 μg revealed that 24% was found in the periphery at death, confirming leakage from the brain. The HC-3 found in the body (18.2 μg or 622 μg/kg) was four times the amount (4.5 μg, 150 μg/kg) which caused 100% mortality when given intraperitoneally. This suggested that HC-3 could be causing death at a peripheral site after i.c.v. administration. This hypothesis was confirmed when the amount of hemicholinium-3 (18.2 μg or 622 μg/kg) found in the body was given systematically. The time to death (11.3 min) was not significantly different from the time to death (15.4 min) after 75 μg i.c.v. (Table 1).

Since HC-3 appeared to be exerting its toxicity peripherally, various peripheral acting neuronal agents were given simultaneously with HC-3 in an attempt to alter the toxicity. Previous studies indicated that HC-3 affects neuromuscular function (Elmquist & Quastel, 1965) by blocking the uptake of choline (Gardiner, 1961) and therefore ACh synthesis (Freeman *et al.*, 1975). In agreement with a peripheral mechanism of toxicity for HC-3, (+)-tubocurarine significantly increased the toxicity and neostigmine decreased the toxicity of HC-3.

In summary, the results of this study do not support the conclusion of previous studies (Schueler, 1955; Kase & Borison, 1958; Borison, 1961; Quastel & Curtis, 1965; Clement, 1978) which suggested a central site of lethal action of HC-3. HC-3 was not observed in, nor did it deplete ACh in the mouse brain after systemic administration. The dose-response curve after intraventricular administration demonstrated that HC-3 was not potent in causing central lethality and that leakage into the periphery had occurred. These results suggested that HC-3 manifests its toxicity primarily in the periphery.

References

BARNES, C.D. & ELTHERINGTON, L.G. (1966). *Drug Dosage in Laboratory Animals.* Berkeley: Univ. Calif. Press.

BORISON, H.L. (1961). Respiratory depressant effect of HC-3. *Fedn. Proc.*, **20**, 594–599.

BERTOLINI, A., GREGGIA, A. & FARRARI, W. (1967). Atropine-like properties of hemicholinium-3, *Life Sci.*, **6**, 573.

CLEMENT, J.G. (1978). Effect of ethanol, pentobarbital, and morphine on the toxicity of hemicholinium-3 and d-tubocurarine in mice. *Archs. Int. Pharmacodyn. Thér.*, **236**, 60–65.

COLLIER, B. & MACINTOSH, F.C. (1969). The source of choline for acetyl-choline synthesis in a sympathetic ganglion. *Can. J. Physiol. Pharmac.*, **47**, 127–135.

DE BALBIAN-VERSTER, F., ROBINSON, C.A., HENGEVELD, C.A. & BUSH, E.S. (1971). Freehand cerebroventricular injection technique for unanesthetized rats. *Life Sci.*, **10**, 1395–1402.

DOMER, F.R. & SCHUELER, F.W. (1960). Synthesis and metabolic studies of C^{14} labelled hemicholinium number three. *J. Am. pharm. Ass.* (Sci. ed.) **49**, 553–580.

DOMINO, E.F., CASSANO, G.B. & PLACIDI, G.F. (1974). Autoradiographic distribution of ^{14}C-hemicholiniuma 3 in mouse whole body and dog brain. *J. Pharmac. exp. Ther.*, **188**, 77–85.

DOMINO, E.F., MOHRMAN, M.E., WILSON, A.E. & HAARSTAD, V.B. (1973). Acetylsecohemicholinium-3, a new

choline acetyltransferase inhibitor useful in neuropharmacological studies. *Neuropharmac.*, **12**, 549–561.

DREN, A.T. & DOMINO, E.F. (1968). Effects of hemicholinium (HC-3) on EEG activation and brain acetylcholine in the dog. *J. Pharmac. exp. Ther.*, **161**, 141–154.

ELMQUIST, D. & QUASTEL, D.M.J. (1965). Presynaptic action of hemicholinium at the neuromuscular junction. *J. Physiol.*, **177**, 463–482.

FREEMAN, J.J., CHOI, R.L. & JENDEN, D.J. (1975). The effect of hemicholinium on behavior and on brain acetylcholine in the rat. *Psychopharmac. Commun.*, **1**, 15–27.

FREEMAN, J.J., MACRI, J.R., CHOI, R.L. & JENDEN, D.J. (1979). Studies on the behavioral and biochemical effects of hemicholinium *in vivo*. *J. Pharmac. exp. Ther.*, **210**, 91–97.

GARDINER, J.E. (1961). The inhibition of acetylcholine synthesis in brain by a hemicholinium. *J. Biochem.*, **81**, 297–234.

GIARMAN, N.J. & PEPEU, G. (1962). Drug induced changes in brain acetylcholine. *Br. J. Pharmac. Chemother.*, **19**, 226–234.

GUYNET, P., ROSSIER, J., BEAUJOUAN, J. & GLOWINSKI, J. (1973). Effect of sodium, hemicholinium-3, and antiparkinson drugs on 14C-acetylcholine synthesis and 3H-choline uptake in rat striatal synaptosomes. *Brain Research*, **62**, 523–529.

HALEY, T.J. & MCCORMICK, W.G. (1957). Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br. J. Pharmac. Chemother.*, **12**, 12–15.

HEMSWORTH, B.A. (1971). The effects of a hemicholinium analogue, HC-15, on neuromuscular transmission. *Euro. J. Pharmac.*, **15**, 91–100.

JOPE, R.S. & JENDEN, D.J. (1979). Dimethylaminoethanol (Deanol) metabolism in rat brain and its effect on acetylcholine synthesis. *J. Pharmac. exp. Ther.*, **211**, 472–479.

KASE, Y. & BORISON, H.L. (1958). Central respiratory depressant action of 'hemicholinium' in the cat. *J. Pharmac. exp. Ther.*, **122**, 215–233.

KOSH, J.W., SMITH, M.B., SOWELL, J.W. & FREEMAN, J.J. (1979). Improvements on the gas chromatographic analysis of acetylcholine and choline. *J. Chromatog.*, **163**, 206–211.

LONGO, V.G. (1958). Action of hemicholinium No. 3 on phrenic nerve action potentials. *J. Pharmac. exp. Ther.*, **122**, 45A.

LONGO, V.G. (1959). Actions of hemicholinium No. 3 on phrenic nerve action potentials. *Archs. Int. Pharmacodyn.*, **119**, 1–9.

MACINTOSH, F.C., BIRKS, R.I. & SASTRY, P.B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature*, **178**, 1181.

MADDEN, J. & MITCHELSON, F. (1975). The interaction of hemicholinium-3 (HC-3) with cholinomimetics and atropine. *Eur. J. Pharmac.*, **32**, 17.

MARSHALL, I.G. (1969). The effects of some hemicholinium-like substances on the chick biventer muscle preparation. *Eur. J. Pharmac.*, **8**, 204.

METZ, B. (1960). Correlation between respiratory reflex and acetylcholine content of pons and medula. *Am. J. Physiol.*, **202**, 80–82.

QUASTEL, D.M.J. & CURTIS, D.R. (1965). A central action of hemicholinium. *Nature*, **208**, 192–193.

ROBINSON, C.A. (1970). *Modification of acetylcholine and choline metabolism in the brain by HC-3.* Ph.D. Dissertation, Vanderbilt University, Nashville.

ROBINSON, C.A., HENGEVELD, C.A. & DE BALBIAN-VERSTER, F. (1969). Improved polyethylene cannulation technique. *Physiol. Behav.*, **4**, 123–124.

SCHUELER, F.W. (1955). A new group of respiratory paralyzants. I. The 'hemicholiniums'. *J. Pharmac. exp. Ther.*, **115**, 127–143.

SCHUELER, F.W., LONGO, V.G. & BOVET, D. (1954). Un nuovo gruppo di parolivzanti centrali. *Arch. Ital. Sci. Farmacol.*, **4**, 328–331.

SHAW, G.G. (1974). Some shortcomings of direct intraventricular injection in mice. *Br. J. Pharmac.*, **50**, 603–605.

SQUIRE, L.R., GLICK, S.D. & GOLGFAR, J. (1971). Relearning at different times after training as affected by centrally and peripherally acting cholinergic drugs in the mouse. *J. comp. Physiol. Psych.*, **74**, 41–45.

YAMAMURA, H.I. & SNYDER, S.H. (1973). High affinity transport of choline into synaptosomes of rat brains. *J. Neurochem.*, **21**, 1355–1374.

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