Pharmacological Studies of Norphenyl Hemicholinium 3

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Norphenyl HC-3 (NP-HC-3), an analog of HC-3 in which the biphenyl nucleus is replaced by a monophenyl moiety, has been synthesized. NP-HC-3 has been shown to resemble the parent compound in most of its pharmacological properties. Its LD_{50} in mice was found to range from 45–680 mcg./Kg. NP-HC-3 in low doses induced a slowly progressive respiratory paralysis. In higher doses the neuromuscular blocking action was antagonized by choline. Neostigmine reversed only the effects observed with low doses of NP-HC-3. NP-HC-3 did not have adrenergic, antihistaminic, analgesic, or local anesthetic activity.

STRUCTURE-ACTIVITY relation studies concerning analogs of hemicholinium number 3 (HC-3) have been carried out by many investigators (1–4). The structure-activity relationships and actions on the peripheral nervous system of some of these analogs have been reviewed by Long (5). The hemicholiniums are characterized by a high toxicity which is slow in onset at minimal lethal doses. They interfere with cholinergic transmission at such sites as nerve-skeletal muscle junctions, autonomic ganglia, and postganglionic parasympathetic endings (6). To date HC-3 remains the most active of the analogs studied for the over-all hemicholinium-like action. The earlier modifications of the HC-3 molecule involved alterations in the cationic moiety and/or the biphenyl nucleus. Schueler (1) found that the phenacyl analog (*i.e.*, half of the parent compound) was inactive. The purpose of this paper is to discuss the synthesis and pharmacology of an analog of HC-3 in which the biphenyl nucleus has been replaced by a monophenyl moiety. This compound hereafter will be referred to as norphenyl-HC-3 (NP-HC-3).

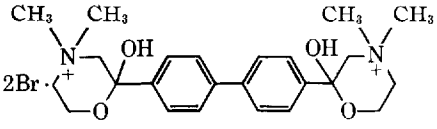
METHODS

Chemical Procedure

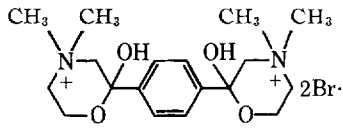
p-Bis(bromoacetyl)benzene was prepared according to the method described by Krohnke and Vogt (7) from 1,4-diacetylbenzene. The product was recrystallized from tetrahydrofuran in the form of stout hexagonal prisms, m.p. 178° (lit. value m.p. 177–178°).

1,4-Bis(1,1-dimethyl-3-hydroxy-3-morpholinyl) Benzene, Dibromide (NP-HC-3).—*p*-Bis-(bromoacetyl) benzene, 10 Gm. (0.031 mole), was dissolved in 200 ml. of boiling tetrahydrofuran. The resulting solution was treated with activated carbon[1] and filtered hot under suction, through diatomaceous earth supported on a sintered-glass funnel. β-Dimethylaminoethanol, 57 ml. (0.05 mole), was added to the hot filtrate, and the mixture was allowed to cool to room temperature. The resulting white precipitate was collected on a filter, washed well with tetrahydrofuran, and recrystallized from a mixture of absolute ethanol and methanol (3:2). The product, collected on a filter, washed well with diethyl ether, and dried over phosphorus pentoxide in a vacuum desiccator, afforded 10.50 Gm. (67.2%) of white crystals, m. p. 228° dec.

Anal.—Calcd. for $C_{18}H_{30}Br_2N_2O_4$: C, 43.39; H, 6.06; N, 5.62. $C_{18}H_{30}Br_2N_2O_4 \cdot H_2O$: C, 42.17; H, 6.37; N, 5.47.[2] Found: C, 42.52; H, 6.34; N, 5.54.[3]



HC-3



NP-HC-3

Pharmacological Procedures

Toxicity Studies in Mice.—Two strains of albino mice (Swiss Webster and $1CR^{CD-1}$) of both sexes, each weighing 20 ± 2 Gm. in groups of 20 were used for each dose of NP-HC-3. The drug was administered intraperitoneally in a volume of 0.01 ml./Gm. body weight in normal saline. In one experiment two LD_{50} determinations were made on the same day, one in the morning and the other in the late afternoon. The LD_{50} was computed by

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[1] Marketed as Norit by American Norit Co., Inc., Jacksonville, Fla.

[2] VBH and FWS (unpublished data) HC-3 has been found to crystallize in variable hydration states. NP-HC-3, as well as HC-3, is very hygroscopic before recrystallization. The water of crystallization is very difficult to remove from HC-3. Thus, it is felt that the NP-HC-3 could have picked up water before and retained it during the recrystallization.

[3] The chemical analysis was performed by Alfred Bernhardt Microanalytical Laboratories, Mulheim, Germany.

means of the probit-log dose method (8). The animals were observed for signs which differed from those of control animals, and the viscera were examined post-mortem in a few cases.

Antagonism Studies in Mice.—Swiss Webster albino mice of both sexes (20 ± 2 Gm. each) were used. All drugs were injected intraperitoneally in a volume of 0.01 ml./Gm. body weight in normal saline. Three groups of 10 mice received 1 mg./Kg. of NP-HC-3 followed 1 min. later by a second injection of saline, 10 or 20 mg./Kg. of choline. Three groups of 5 mice were given 1–3 mg./Kg. NP-HC-3 followed 1 min. later by saline, 150 or 300 mcg./Kg. of neostigmine.

Blood Pressure-Neuromuscular Preparation.—In this series of experiments, 5 mongrel dogs (9–15 Kg.), 5 albino rabbits (2–3 Kg.), and 2 chickens (2–3 Kg.) of either sex were used. All the animals were anesthetized with sodium pentobarbital (35 mg./Kg., i.v.). The blood pressure was recorded from cannulated arteries connected *via* a Statham pressure transducer to a Grass model 5-DWC polygraph. Heparinized saline (50 u./ml.) was used as an anticoagulant in this system. The Achilles tendon was isolated and tied with a thin copper wire, about an inch above the joint, sectioned distally, and attached to a force-transducer (model FT-03). The muscular contractions were recorded on the polygraph. The ipsilateral sciatic nerve was isolated, sectioned proximally, and the peripheral stump stimulated with a bipolar, shielded, platinum electrode, using a Grass stimulator (model S4G) so as to obtain maximal muscular contractions. The leg was immobilized with an iron pin which passed through the knee joint while the foot was clamped to a rigid frame. The trachea was exposed, cannulated, and artificial respiration was used whenever necessary. The femoral vein of the dog, the jugular vein of the chicken, and the ear vein of the rabbit were cannulated to permit drug administration. In the dog and the rabbit, blood pressure recordings were obtained from the right carotid artery, whereas in the chicken the femoral artery was used for this purpose. In both the dog and the rabbit the left vagus was isolated, ligatured, sectioned centrally, and the distal portion stimulated with a Mallory model 12 Rs6D inductorium.

Two chickens (2.5 Kg. male) were prepared as described above. In addition, the branch of the femoral artery feeding the gastrocnemius muscle was isolated, and a loose loop of thread was placed around it. A side branch was cannulated in the direction of blood flow to permit the injection of acetylcholine.

TABLE I.—BLOOD PRESSURE–NEUROMUSCULAR PREPARATION

No.	Wt., Kg.	Frequency of Stimulation/sec.	Dose, mg./Kg.	Onset of Actions, min.	% Neuromuscular Blockade	Duration of Blockade, min.	Remarks
				Dogs			
1	14.1	2	0.1		0	...	
			1.0	15	100	34	Died of respiratory failure at this time
2	10.0	2	0.3	10	10	30	
			0.7	1.5	50	55	
			1.0	1.0	90	80	
			1.3	1.0	95	95	
			1.5	0.5	97.5	Indefinite	Blockade not reversed even after 2 hr.
3	12.0	2	0.7	20	<10	<10	
		2	1.0	25	37	60	
		4	1.0	3	76	120	
		2	1.0	6	42	65	
4	9.1	2	0.7	10	90	45	
		4	0.7	1.5	90	>180	
5	10.0	2	0.7	20	50	30	
		4	0.7	...	...		Died of respiratory failure after this dose
				Rabbits			
1	3.2	0.4	1	13	100	105	
2	2.5	0.4	0.6				
			0.6	10	30	35	
3	2.5	0.4	0.6	4	100	60	Died before recovery
4	2.6	2.0	0.6	15	98	120	
5	2.0	2.0	0.3	...	0	...	
			0.5	...	0	...	
			0.7	10	50	45	
				Chickens			
1	2.5	0.4	0.3	...	0	0	
			0.6	...	0	0	
			1.2	2	65	60	
2	2.6	0.9	1.0	15	100	30	Died during total blockade

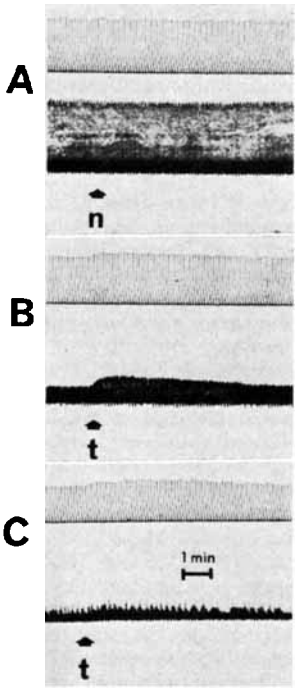

Fig. 1.—Bilateral contractions of the gastrocnemius muscles of the chicken. In the upper tracing the rate of supramaximal electrical stimulation of the ipsilateral sciatic nerve was 0.2/sec. The rate of stimulation in the lower tracing was 0.8/sec. In A, NP-HC-3 (1 mg./Kg.) was given intravenously (n). In B, the first injection of TEA (3 mg./Kg. i.v.) was given. In C, the fourth dose of TEA (3 mg./Kg., i.v.) was given.

Bilateral sciatic-gastrocnemius preparations were done with 3 rabbits and 2 chickens. The arrangement of the apparatus was the same as previously described except that the contractions of both the gastrocnemius muscles of each animal were recorded. One of the muscles was stimulated at a low frequency (0.2–0.4/sec.), and the other was stimulated at varying, higher frequencies. The blood pressure of the chickens was not recorded.

RESULTS

Toxicity Studies in Mice.—About 5 min. after the administration of a 700 mcg./Kg. dose of NP-HC-3 the animals exhibited dyspnea, exophthalmos, and ataxia. Some of the animals exhibited a Straub tail reaction. Then clonic convulsions developed and they stood on their hind limbs. Nearly half of them fell on their sides and died within 15–20 min. Survivors were apparently normal after 2 hr.

Irrespective of the increment of the doses above the lethal levels, a minimum of about 5 min. elapsed before the observable onset of action. At autopsy the heart was still beating, and the intestinal motility appeared to be stimulated slightly more than in control animals.

The LD_{50} (i.p.) in two different batches of ICR-CD-1 albino mice was 680 mcg./Kg. and 320 mcg./Kg. The LD_{50} (i.p.) of NP-HC-3 in a strain of Swiss Webster albino mice was 45.5 mcg./Kg. in the morning and 46.3 mcg./Kg. in the late evening of the same day. This is in contrast to the large variation which previously has been found with HC-3.

Antagonism Studies.—All mice receiving 1 mg./Kg. of NP-HC-3 died within 30–35 min. Three of ten given 10 mg./Kg. of choline died at 10–15 min. Only one of ten treated with 20 mg./Kg. of choline died in 15 min.

A dose of 150 mcg./Kg. of neostigmine protected all mice treated with 1 mg./Kg. of NP-HC-3. All mice died which were given 2 or 3 mg./Kg. of NP-HC-3. In these animals neostigmine produced no marked parasympathomimetic activity. Mice given 300 mcg./Kg. of neostigmine died within 3 min., and exhibited excessive salivation, lacrimation, urination, and defecation (SLUD syndrome). Animals which received this dose of neostigmine 1 min. after a 1 mg./Kg. of NP-HC-3 were slightly ataxic after 20 min. and did not exhibit the SLUD syndrome.

Blood Pressure-Neuromuscular Preparation.—Doses of NP-HC-3 from 0.1–1.5 mg./Kg. did not have any effect on the blood pressure of the dog, rabbit, or chicken. Also, it did not modify the control responses produced by vagal stimulation and double carotid occlusion of injections of acetylcholine, epinephrine, or histamine.

The results found with the neuromuscular preparations may be seen in Table I. Doses of 1 mg./Kg. or less exerted varying degrees of blockade. Increasing the rate of stimulation increased the amount of blockade as well as its duration, *e.g.*, dog 3. Choline (5–20 mg./Kg. i.v.) reversed the neuromuscular blockade either partially or completely. Four 3 mg./Kg. (i.v.) doses of tetraethylammonium bromide given at 3-min. intervals to a rabbit during partial neuromuscular blockade with NP-HC-3 caused a partial reversal of the blockade with the initial dose. The amount of reversal

TABLE II.—EFFECTS OF INTRAVENOUS NP-HC-3 (1 mg./Kg.) ON BILATERAL SCIATIC NERVE-GASTROCNEMIUS PREPARATIONS IN RABBITS AND CHICKENS

Animal	Wt., Kg.	Side	Frequency of Stimulation/sec.	Onset of Action, min.	% Neuromuscular Blockade	Duration of Blockade, min.	Choline, 10 mg./Kg.	Neostigmine, 1–3 mg./Kg.
Rabbit 1	2.6	Left	0.8	15	100	30	Completely reversed	No change
		Right	0.4	25	10	...	No change	Potentiation
Rabbit 2	4.0	Left	3.0	30	50	48	Partially reversed	No change
		Right	0.4	40	10	20	No change	Potentiation
Rabbit 3	3.4	Left	0.4	9	70	70	Partially reversed	...
		Right	0.8	4	90	85	Partially reversed	...
Chicken 1	3.4	Left	3.0	7	90	30	Partially reversed	...
		Right	0.4	20	50	25	No change	...
Chicken 2	3.0	Left	0.2	40	50	60	Partially reversed	...
		Right	0.8	25	95	120	Partially reversed	...

decreased with successive doses until no effect was observed (Fig. 1).

The effect of frequency of stimulation on the intensity and duration of neuromuscular blockade and the action of choline and neostigmine are listed in Table II. Here again, increasing the frequency of stimulation increased the rate of onset of neuromuscular blockade as well as the strength and duration of the blocking action. In rabbits, neostigmine (0.1 mg./Kg.) potentiated the strength of contraction on a side being stimulated at 0.4/sec. and had almost no effect on the side being stimulated at 3/sec. (Fig. 2, B). The same effect was seen with edrophonium (Fig. 2, C), in which case the potentiated height of contraction was more than twice that of the control. Following these 2 drugs, choline administration reversed completely the neuromuscular blockade in the leg being stimulated more rapidly and had no effect on the other side (Fig. 2, D). The contraction produced by a close-arterial injection of 6 mcg. of acetylcholine on the unstimulated muscle of a chicken was completely abolished 30 min. after the i.v. administration of 1 mg./Kg. of NP-HC-3 (Fig. 3 A–C). This was at the onset of the neuromuscular blockade. When the electrical stimulation was resumed following the administration of acetylcholine, there was an initial increase in the strength of the muscular contraction (Fig. 3, D). Ten micrograms of acetylcholine caused a slight depolarization which lasted about 0.5 min. During complete neuromuscular blockade either 6 or 10 mcg. of acetylcholine administered close-arterially during the electrical stimulation resulted in a burst of weak contractions. A dose of 10 mg./Kg. of choline administered i.v. resulted in a series of contractions which were 14% of control and which lasted for a period of 6 min. (Fig. 3, F).

Miscellaneous Studies.—NP-HC-3 did not have any effect on the rat or guinea pig ilia or on responses produced by test doses of acetylcholine, epinephrine, and histamine. It did not exhibit any non-narcotic analgesic activity in mice studied according to the method of Koster *et al.* (9) or any narcotic analgesic activity in mice tested according to the method described by D'Amour and Smith (10). There was also no topical or infiltrative local anesthetic activity found using the rabbit.

DISCUSSION

NP-HC-3 was synthesized in an attempt to investigate the minimum requirements for the hemicholinium-type of activity and also to gain more insight into the mechanism of action of the hemicholiniums. NP-HC-3 ranks next to HC-3 itself in potency among the compounds previously studied for the hemicholinium-type of activity. The primary toxic manifestations of NP-HC-3 are a slowly progressive respiratory depression and muscular weakness. As in the case of HC-3 the LD_{50} in mice varied widely with different batches of mice used, and the observed effects occurred after a latent period which was independent of the dose. On a molar basis the LD_{50} of NP-HC-3 was about 3 times that of the parent compound, HC-3.

The toxicity of both NP-HC-3 and HC-3 is antagonized by choline and neostigmine. As with HC-3 (11) there was a rough correlation among the lethal dose with NP-HC-3, the size of the animal, and the time required for the onset of action.

The neuromuscular blockade produced in the sciatic nerve-gastrocnemius muscle of the rabbit, chicken, and dog was slow in onset and could be augmented by increasing the rate of stimulation. The duration of the blockade was from 30 min. to 2 hr. With successive doses, the time of onset decreased, and the duration of the neuromuscular blockade increased. Similar effects were observed with curarization in humans by Bush and Baraka (12). The apparent potentiation of a second dose of tubocurarine had been discussed by Levy (13). He established a quantitative relationship between the duration of action and the concentration of the drug in successive doses. The observations made with NP-HC-3 are compatible with Levy's hypoth-

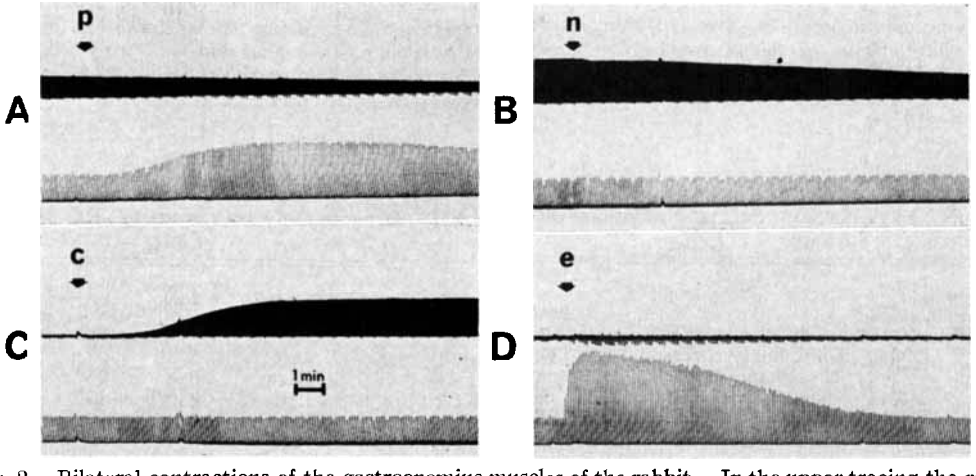

Fig. 2.—Bilateral contractions of the gastrocnemius muscles of the rabbit. In the upper tracing the rate of supramaximal electrical stimulation of the ipsilateral sciatic nerve was 3/sec. The rate of stimulation in the lower tracing was 0.4/sec. In A, NP-HC-3 (n) 1 mg./Kg., i.v. was given. In B, neostigmine (p), 0.05 mg./Kg., i.v. was given. In C, edrophonium (e), 0.2 mg./Kg., i.v. was given. In D, choline (c), 10 mg./Kg., i.v. was given.

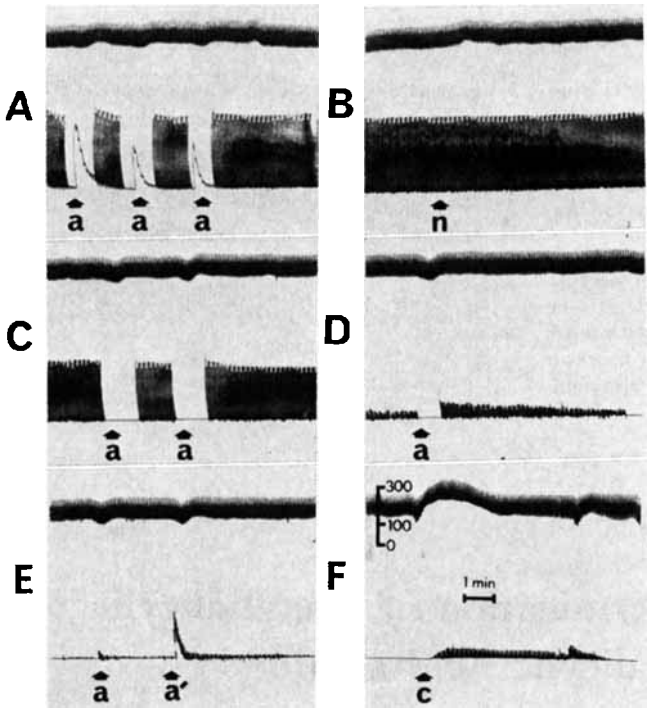

Fig. 3.—Femoral arterial blood pressure and gastrocnemius muscular contractions of the chicken. In A, three close-arterial injections of acetylcholine (a) 0.006 mg./Kg., i.v. In B, NP-HC-3 (n), 1 mg./Kg., i.v. was given. In C, D, and E acetylcholine was given in doses of 0.006 mg./Kg. (a) and 0.01 mg./Kg. (a^1) i.v. In F, choline (c), 10 mg./Kg., i.v. was given.

esis. The neuromuscular blockade produced by high frequency stimulation could be reversed partially, and often completely, with choline and not with neostigmine. However, with muscles being stimulated at a lower frequency, there was no antagonism with choline, whereas neostigmine caused a considerable augmentation in the height of contraction. This may be explained by assuming that during the total blockade seen during high frequency stimulation, all of the acetylcholine stores had been depleted. Thus, even relatively high concentrations of the indirect-acting neostigmine were ineffective in increasing the acetylcholine concentration to an effective level. On the other hand, choline, being the precursor of acetylcholine, replenished the reserves. During low frequency stimulation the acetylcholine stores were only partially depleted.

The temporary reversal observed with initial doses of tetraethylammonium (TEA) during total neuromuscular blockade with high frequency stimulation, and the ineffectiveness of subsequent doses of TEA, has been observed previously by Volle (14) with HC-3. TEA has been shown to be a releaser of acetylcholine from presynaptic nerve endings (15). This is suggestive of a situation in which NP-HC-3 or HC-3 inhibit the synthesis of acetylcholine in presynaptic nerves, and the effect observed with the initial doses of TEA was a facilitated release of the residual acetylcholine. The effect of TEA on the neuromuscular blockade produced by NP-HC-3 and HC-3 should also be reconsidered in the light of recent findings by Bhatnager *et al.* (16). They found that TEA and a variety of other quaternary ammonium compounds acted as inhibitors of acetylcholine synthesis in nervous tissue. The apparent paradox posed by the observations of Volle, Bhatnager, and ourselves may be resolved by pointing out that quaternary ammonium compounds such as HC-3, NP-HC-3, and TEA, may act in at least two ways: (*a*) they may either increase the fragility of the synaptic vesicles, or (*b*) they may inhibit formation of acetylcholine.

It is possible that these quaternary ammonium compounds act like detergents and cause an emulsification of the vesicular membranes. Under such conditions the acetylcholine formed would be exposed to immediate hydrolysis by acetylcholinesterase. In addition, the rate of transport of these quaternary ammonium compounds to the site of acetylcholine synthesis may be altered during varying levels of nervous activity, *e.g.*, resting *versus* high frequency stimulation. Perhaps this may be the reason why the increase in frequency of stimulation lowered the effective neuromuscular blocking doses and accelerated the onset and increased the duration of action.

The lack of response to the close-arterial injections of acetylcholine in the unstimulated gastrocnemius muscle of the chicken during the course of complete neuromuscular blockade indicates a curare-like effect of NP-HC-3. However, the temporary reversal of the neuromuscular blockade seen when acetylcholine was administered close-arterially during or immediately prior to electrical stimulation suggests an additional mechanism of action, *i.e.*, the injected acetylcholine is taken up by the nerve and is then available for release in response to stimulation. The release of the newly acquired acetylcholine from the nerve ending may require extraneous stimulation such as electrical impulses or TEA. The lack of effect of neostigmine during total neuromuscular blockade in preparations

stimulated at high frequencies could reflect either a deficiency of acetylcholine release or that the end plate receptors are already occupied. Observation that the blockade is reversed by acetylcholine injected close-arterially during stimulation indicates that the latter situation does not obtain. Therefore, since the receptors can still respond to administered acetylcholine, the evidence is in favor of a presynaptic, rather than a postsynaptic, mechanism of action.

NP-HC-3 may be assumed to act, at least in part, by decreasing the presynaptic supply of acetylcholine. The exact mechanism by which this is caused has not been elucidated. Replacement of the biphenyl by the monophenyl nucleus has not altered the hemicholinium-like activity, although there is a decrease in the toxicity.

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Fluorometric Determination of Acetylsalicylic Acid and Salicylic Acid in Blood

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A useful micro-method has been developed for the paper chromatographic separation of acetylsalicylic acid and salicylic acid with subsequent determination of each compound by fluorometry from a 0.1-ml. sample of capillary blood. This method permits frequent determinations of both acetylsalicylic acid and salicylate blood levels on the same subject. The accuracy and precision of the micro-method has been studied. *In vivo* studies utilizing the method emphasized the validity of results obtained by a rapid micro-method of analysis.

MANY METHODS for the determination of salicylates in blood and in plasma have been described in the literature. The generally accepted methods of Brodie (1) and Routh (2) involve the extraction of the salicylate from the blood sample and the determination of the concentration colorimetrically by measuring the absorbance of an iron complex. However, Saltzman (3) and Chirigos (4) have described the determination of salicylate in biological tissues by measurement of the characteristic fluorescence of the salicylate ion on exposure to ultraviolet light.

The quantity of acetylsalicylic acid in the biological tissues was estimated from the difference between "free" salicylate and "total" salicylate, and conjugated salicylate being considered to be acetylsalicylic acid (5). Mandel (6) has reported a paper chromatographic procedure for the separation of acetylsalicylic acid from salicylic acid in a plasma sample, and their separate determination fluorometrically. Recently, Nikelly (7) described the gas chromatographic determination of acetylsalicylic acid in the presence of salicylic acid.

Although the information obtained from any of the above methods is valuable, there does not seem to be general agreement among investigators about the levels of acetylsalicylic acid in the blood after taking aspirin. Since the time span of the analgesic effect of aspirin (2–4 hr.) appears to be more closely related to the time that the acetylsalicylic acid persists in the blood, the blood level of acetylsalicylic acid would seem to be a critical measure of the potential analgesic effectiveness of an aspirin formulation. Thus, for the *in vivo* study of acetylsalicylic acid blood levels it is desirable to have a micro-method requiring small volumes of blood for the repeated sampling required to observe simultaneously sustained blood levels of acetylsalicylic acid and salicylic acid. The method should also be fast, practical, and accurate.

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