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PHARMACOLOGIC TEST PREPARATIONS THAT DISTINGUISH ACETYLCHOLINE AND ACETYL-*l*-CARNITYL COENZYME A

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Abstract—Previous work had shown that the material with acetylcholine-like activity extractable from the brains of narcotized rats was indistinguishable from acetylcholine with certain pharmacologic test preparations. Recently the material responsible for this acetylcholine-like activity has been analyzed chemically and found to be acetyl-*l*-carnityl CoA; hence it was important to find pharmacologic test preparations that might distinguish between acetyl-*l*-carnityl CoA and acetylcholine. It was found that, through the relative rates of hydrolysis of these substances by the frog heart and frog rectus preparation, we can distinguish these two substances.

CHANG and Gaddum[1] showed that the method of parallel bioassay enabled the identification of acetylcholine from other choline esters. Recently Hosein[2] provided evidence that the material with acetylcholine-like activity in narcotized rat brain extracts was mainly acetyl-*l*-carnityl CoA. It therefore seemed likely that this substance could be distinguished from acetylcholine by pharmacologic test preparations.[1] Bremer,[3] Marquis and Fritz,[4] and Pearson and Tubbs[5] have assayed the acetyl group of acetyl-*l*-carnitine in brain, heart and liver with the enzyme carnitine acetyltransferase. Fritz[6] has also shown that acetyl-*dl*-carnitine administered to animals produced convulsions and other cholinergic effects which were similar to those obtained by Feldberg and Sherwood,[7, 8] who performed similar experiments with acetylcholine. Weger[9] in 1936 had observed that the pharmacologic activity of acetyl-*l*-carnitine was indistinguishable from that of acetylcholine. He suggested that acetyl-*l*-carnitine might well function as a transmitter substance *in vivo*. Dallemagne *et al.*[10] and Hosein and Koh[11] have described the acetylcholine-like activity of acetyl-*l*-carnitine, and these authors have shown that this substance is less active than acetylcholine on the eserinized frog rectus preparation. However, consideration should be given to the fact that acetyl-*l*-carnitine was tested—not acetyl-*l*-carnityl CoA, a diester of carnitine. Strack and Fosterling[12, 13] and Kato[14] have shown that further esterification of the monoester of carnitine enhances cholinergic activity.

Hosein and Koh[11] have described several test preparations that cannot distinguish mixtures of acetylcholine, acetyl-*l*-carnitine, and acetyl-*l*-carnityl CoA obtained from narcotized rats. The present investigations were designed to search for pharmacologic test preparations that could be distinguishing test objects for these naturally occurring materials.

METHODS

Rats were injected intraperitoneally with pentobarbital (65 mg/kg). After a 30-min period of deep narcosis, the animal was decapitated, the brain excised, and the material with acetylcholine-like activity extracted with trichloroacetic acid and chromatographed in the water-saturated butanol system as previously described.[15, 16] Two experiments using (A) the frog heart and (B) the frog rectus are described.

(A) A stock solution of acetylcholine chloride (Merck) and the eluate of the band of the chromatogram with R_f 0·5–0·7 known to contain acetyl-*l*-carnityl CoA[2] were assayed with the sensitized frog rectus preparation,[15, 17] with the precautions described by Feldberg.[18] The volume of the muscle bath was 3·0 ml. Brain extract or reference standard was added to hydrolysates of the eluate to produce stock solutions (i) and then assayed with the sensitized frog rectus preparation. These stock solutions (i) were then diluted to produce solutions (ii), each of which now contained the equivalent of 10 μg acetylcholine chloride/ml frog rectus assay.

The central nervous system of the frog was destroyed by pithing the animal. The heart was exposed, the vena cava cannulated, and a small slit made in the bulbus arteriosus to allow escape of the perfusate. A small hook attached to the apex of the ventricle was attached by a fine thread to a force transducer connected to a M-5P Mini-Polygraph (Gilson Medical Electronics). The flow of Locke's perfusion solution entering the heart was controlled by a Baxter R-41 administration set. An insertion was made in the rubber tubing leading to the cannula, and all injections of materials to be tested were always made at this particular point. These injections rarely exceeded 0·1 ml and were made with a 1-ml "tuberculin" syringe.

The *in situ* frog heart was perfused with Locke's solution, pH 7·8,[19] which was prepared with (10^{-6} M) or without eserine, depending on the particular experiment.

Atropine antagonism of heart action was investigated by infusing 0·1 ml atropine sulfate (10^{-6} M) at the injection site. Immediately after this injection, an amount of either acetylcholine or acetyl-*l*-carnityl CoA, previously found to be effective in stopping the heart, was injected, and the effects on the heart rate, if any, were then noted.

(B) The equivalent acetylcholine-like activity in each stock solution (ii) was adjusted to elicit a contraction 2 cm high in 90 sec with the uneserinized frog rectus preparation. The bath fluid (Locke's solution) was then replaced with Locke's solution containing 10^{-5} M eserine and the muscle preparation allowed to relax for 1 hr. Aliquots of solution (ii) of the extract and the standard were then assayed to produce the same response (height of contracture) as observed originally with the uneserinized preparation.

RESULTS

A. Studies of frog heart

The minimal amount of acetylcholine chloride necessary to stop the beat of the uneserinized frog heart was determined by successively perfusing into the preparation increasing concentrations of solutions (ii) containing acetylcholine chloride. This amount of acetylcholine was then compared with the equivalent activity of acetyl-*l*-carnityl CoA (narcotized brain extract) to produce the same effect on the same heart. This procedure was repeated with twenty frog hearts. In several experiments, the order of testing was reversed. The results obtained are recorded in Table 1 and Fig. 1.

It can be seen in Fig. 1 that 1 μg acetylcholine chloride as assayed with the frog rectus preparation was required to stop the beat of the frog heart. These results are in full agreement with those of Boyd and Pathak.[20] This value was three times that of the assayed equivalent acetyl-*l*-carnityl CoA activity required to produce the same effect. The difference was statistically significant ($P < 0{\cdot}001$).

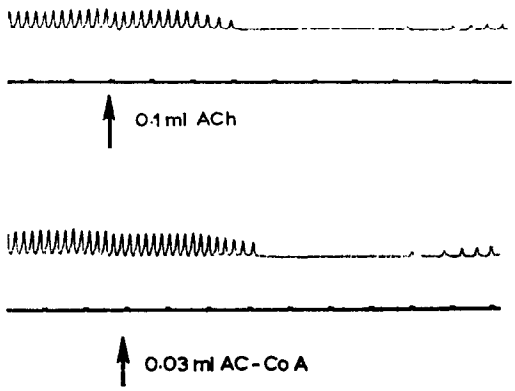

FIG. 1. A comparison of the minimal amounts of acetylcholine and acetylcholine-CoA required to cause heart arrest.

TABLE 1. EFFECT OF ESERINE ON THE MINIMAL AMOUNT OF ACETYLCHOLINE AND ACETYL-*l*-CARNITYL CoA REQUIRED TO ARREST THE BEAT OF THE ISOLATED FROG HEART

Substance	Minimal amount of material required for heart arrest		
	Before eserine	After eserine	Ratio
Acetylcholine	1003 ± 190	250 ± 45	4:1
Acetyl-*l*-carnityl CoA	300 ± 50	250 ± 40	1:1
Ratio	3:1	1:1	

Values expressed as mμg ACh equivalent activity

When these experiments were repeated with eserinized Locke's solution, the extent of protection from cholinesterase destruction offered to these substances differed markedly. The results in Table 1 indicate that the sensitivity of the preparation to acetylcholine increased fourfold, while that to acetyl-*l*-carnityl CoA was virtually unchanged. It should also be observed that, while the ratio of the equivalent acetylcholine-like activities of the two substances was 3 : 1 with the uneserinized preparations, it was identical with the eserinized preparation. The results indicated that, although acetyl-*l*-carnityl CoA may be as active as acetylcholine on most of these preparations sensitized with eserine, there may be limited destruction of it in the uneserinized preparation.

Atropine antagonism of these effects of acetylcholine chloride and of acetyl-*l*-carnityl CoA on the perfused frog heart are shown in Fig. 2.

Hydrolysis of the effective concentrations of both materials was carried out by boiling in alkali (pH 11) for 5 min. The solutions were cooled, neutralized to pH 7·8

with 0·1 N HCl, and again tested on the frog heart. They were now both without effect, indicating that the original discrepancy in effectiveness observed was due to these esters and not to inorganic salts which could have been carried through the extraction and chromatographic procedures.

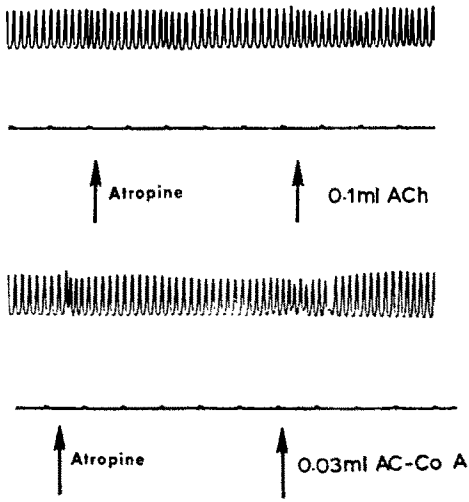

FIG. 2. Antagonism of atropine to the inhibitory effects of acetylcholine and acetyl-*l*-carnityl CoA on the frog heart.

B. Studies of frog rectus abdominis preparation

Material present in the eluate of the band of the chromatogram with R_f 0·5–0·7 was assayed with the uneserinized frog rectus preparation with acetylcholine chloride as reference standard. The acetylcholine-like activity in solutions (ii) contained the equivalent of 1·0 μg acetylcholine chloride/ml; 150 mμg equivalent acetylcholine chloride activity in each of these solutions was required to produce 2 cm contracture with the uneserinized frog rectus preparation. Smaller doses of acetylcholine were tried, but they were ineffective under our particular experimental conditions (bath volume 3·0 ml) in eliciting a response within 90 sec. These results are shown in Fig. 3.

When the preparation was treated with eserine and the experiment repeated with various amounts of each solution, it was observed that 50 mμg acetylcholine chloride on the average caused a contraction between 1·0 and 1·2 cm; 80 mμg acetylcholine chloride or 50 mμg equivalent acetylcholine activity from the narcotized brain extract containing acetyl-*l*-carnityl CoA consistently elicited 2-cm contracture.

In either experiment, the effects of the eluate were abolished by alkaline hydrolysis. Addition of acetylcholine or eluate to such neutralized hydrolysates did not alter the response of the added materials.

DISCUSSION

The results described above show that the material extractable from the brain of narcotized rats, although assayed to the same value as acetylcholine, behaves quite differently from it with eserinized and uneserinized test preparations. These results

support other work by Hosein *et al.*,[21] who observed colorimetrically that the acetylcholine activity in narcotized brain extract was likely due to acetyl carnitinyl-CoA rather than acetylcholine. Earlier, Hosein and Koh[22] had shown that the sensitized frog rectus preparation could not distinguish between acetyl-*l*-carnitine and acetylcholine and between mixtures of these two compounds. Chemical analysis of brain extracts prepared from 50 narcotized rats by Hosein and Koh[23] and Hose in and Orzeck[24]

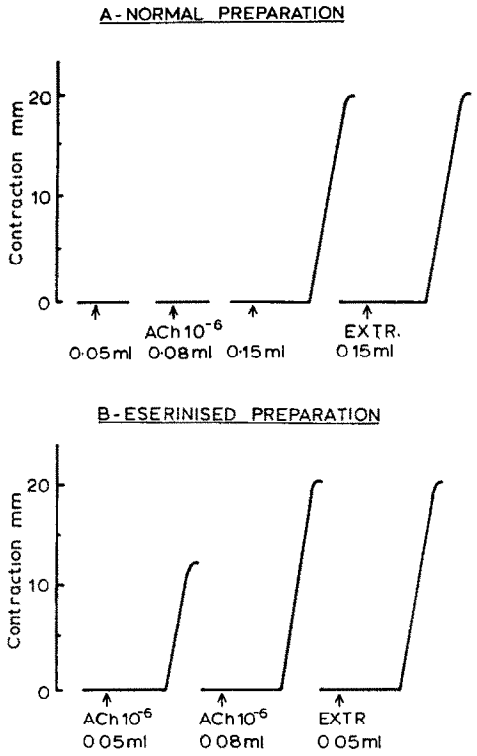

FIG. 3. A comparison of the effects of acetylcholine and acetyl-*l*-carnityl CoA on the normal and eserinized frog rectus preparations.

failed to reveal the presence of acetylcholine. In addition, these authors further showed that when 138 μg acetylcholine chloride (equivalent to the acetylcholine-like activity contained in a sample of narcotized brain extract) was added to that assayed sample, this added acetylcholine could be fully recovered and identified chemically by the melting point of its tetrachloroaurate derivative.[16] There was no such recovery of acetylcholine in the untreated duplicate sample of narcotized brain extract.

The results from microchemical analyses indicated that in narcotized brain extracts acetyl-*l*-carnityl CoA is mainly responsible for the acetylcholine-like activity. Accordingly, acetylcholine and acetyl-*l*-carnityl CoA should be distinguishable by suitable test preparations.[1] The results quoted above showed that by comparing the relative rates of hydrolysis of these two substances by the frog heart and the frog rectus preparation, a method was found that can readily distinguish the two substances. In addition, the results indicated that, if the material with acetylcholine-like activity in

the narcotized brain extract were acetylcholine,[25–27] there should have been no discrepancy between the values obtained with the test preparations, because acetylcholine chloride was the reference standard used in both instances.

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