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SHORT COMMUNICATIONS

Acetylcholine-like activity in sciatic nerve—A re-evaluation

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Parallel bioassays showed that the acetylcholine-like activity in acetone extracts of sucrose homogenates of rabbit sciatic nerves was due not only to acetylcholine (ACh) but to another ester or esters of choline [1]. The ACh-like activity could be demonstrated only in extracts prepared from nerves of animals treated with physostigmine; the ACh-like activity was acid stable and alkali labile, and its actions could be annulled by *d*-tubocurare when tested on the frog rectus abdominis muscle and by atropine when tested on the isolated guinea pig ileum. The ACh-equivalents of the extracts measured on the frog rectus preparation were always greater by a factor of 1.6 than that measured on the guinea pig ileum. Neither an inhibitor nor a potentiator of ACh could be shown. Thus, these results showed that acetone extracts of sucrose homogenates of rabbit sciatic nerve contain, in addition to ACh, another compound(s) that is pharmacologically indistinguishable from a choline ester, and that this compound(s) has greater nicotinic activity than muscarinic activity.

We report here the results of studies undertaken to re-evaluate these early observations. We show that the appearance of the additional choline ester(s) as determined pharmacologically, depends upon the homogenization and extraction procedure, and that the additional choline ester is an artifact.

Adult male Dutch Belted and New Zealand rabbits were used in this study. The animals were anesthetized with sodium pentobarbital, 50 mg/kg, i.p., and after the loss of the corneal reflex, physostigmine sulfate, 1 mg/kg, i.p., was administered. Usually, by 10 min after physostigmine administration, marked salivation, urination and/or defecation occurred; if not, additional physostigmine, 0.25 mg/kg, i.v., was administered. After the signs of cholinesterase inhibition were observed, the animals were killed by injecting air into the marginal ear vein. The major branches of the sciatic nerves were dissected from a region near the spinal cord to their entry into the muscles of the lower limb. The nerves were weighed, minced with scissors and homogenized with 4 vol. of ice-cold 0.32 M sucrose containing 5×10^{-5} M physostigmine sulfate with either a Potter–Elvehjem or a Polytron (Brinkman Instruments, Westbury, NY) homogenizer. Acetone (20 vol.) was added to the homogenate and allowed to mix for 1 hr at room temperature. The particulate matter was removed by centrifugation and the supernatant fraction taken to dryness by evaporation under reduced pressure (final pressure < 1 mm Hg at 22°). The residue was suspended in a small volume of 0.02 M NaH_2PO_4, pH 3.7, and stored at −20° until assayed for ACh on preparations of the frog rectus abdominis muscle and guinea pig ileum [1, 2]. In some experiments, the acid phosphate suspension was further extracted with 9 vol. anhydrous acetonitrile containing 1 g anhydrous $MgSO_4$ and 1 nmole propionylcholine as internal standard, and the ACh present in the acetonitrile extract was determined by pyrolysis–gas chromatography (p.g.c.) [3]. In other experiments, acid extracts of the minced nerves were directly prepared with 0.02 M NaH_2PO_4 [2], and ACh present in the extracts was measured by bioassay.

In agreement with our original observations [1], we found that the ACh-equivalents of acetone extracts of sucrose homogenates prepared with a Potter–Elvehjem homogenizer were greater when assayed on the frog rectus preparation than when measured on the guinea pig ileum. For nerves obtained from New Zealand rabbits, which were used in the original work [1], the parallel bioassay for ACh activity showed a ratio (frog rectus/guinea pig ileum) of 1.65 ± 0.02 ($n = 4$); two experiments on Dutch Belted rabbits gave ratios of 102 and 34. These results are summarized in Table 1. Further, in agreement with our original observations [1] on both the rectus muscle and the ileum, the ACh-like activity in the extracts from both strains was pharmacologically indistinguishable from a choline ester in being acid stable (pH 4, heated for 10 min in a boiling water bath), alkali labile (pH 9, heated for 10 min in a boiling water bath), and antagonized by atropine on the ileum and by *d*-tubocurarine on the rectus muscle. Reassay of the acid boiled extracts on both preparations gave results identical to those obtained for the unboiled samples. The dose–response curves to standard ACh and to the extracts were also parallel on these muscles. Further, repeating the assays after the addition of authentic ACh to the extracts failed to demonstrate the presence of either an inhibitor or a potentiator of ACh; the effects of the extract and of known ACh are additive. The possibility that the higher values for ACh obtained with the rectus muscle are due to the sensitizing effect of acetone [2] is ruled out by our finding that extracts heated at pH 4 at 100° for 10 min give the same value as unheated samples, that the effects of the extract and authentic ACh are additive, and by the very low pressure (< 1 mm Hg) used in drying the initial extract. That a sensitizer such as acetone is not responsible for the higher ACh-equivalents obtained with the frog rectus muscle was established in our initial investigations [1] and confirmed here.

The reason for the extremely high values of ACh activity

Table 1. ACh-like activity in acetone extracts of sucrose homogenates of rabbit sciatic nerve when homogenates were prepared with a Potter–Elvehjem homogenizer

Strain of rabbit	ACh-equivalents (nmoles/g) Frog rectus	Guinea pig ileum	Parallel bioassay ratio *
New Zealand	$12.91 \pm 0.6^{+}$ (4)	$7.6 \pm 0.05^{+}$ (4)	1.69 ± 0.04
Dutch Belted	4100, 2500	40, 73	102, 34

* Ratio: ACh-equivalents on frog rectus/guinea pig ileum.
+ Values are mean ± S.E.M.; number of animals is given in parentheses.

Table 2. ACh-like activity in extracts of sciatic nerve from Dutch Belted rabbits when homogenates were prepared with a Polytron homogenizer

Homogenization medium	ACh-equivalents (nmoles/g) Frog rectus	Guinea pig ileum	Parallel bioassay ratio *
0.02 M NaH_2PO_4	31, 24	31, 25	1.0, 0.9
0.32 M Sucrose†	26, 24	26, 24	1.0, 1.0

* ACh-equivalents on frog rectus/guinea pig ileum.
† Followed by extraction with acetone.

(especially as measured on the frog rectus abdominus muscle) in extracts of nerves from Dutch Belted rabbits as contrasted to those from the New Zealand strain is not known. Pretreatment with physostigmine and the presence of physostigmine in the homogenization medium suggest that a great difference in the activity of the esterases is not a likely cause of the difference. Measurements of choline acetyltransferase activity [4] in nerves from each strain did not reveal significant differences between the two: 5.5 ± 0.5 (range) nmoles/mg of tissue/hr and 5.0 ± 0.4 (range) nmoles/mg of tissue/hr for Dutch Belted and New Zealand strains respectively. The results of other experiments (Table 2) confirm that the levels of ACh activity, as determined on the guinea pig ileum, are greater in sciatic nerves obtained from Dutch Belted than from New Zealand rabbits.

Comparable experiments were then carried out with different homogenization and extraction techniques. The sciatic nerves from four Dutch Belted rabbits were homogenized in either 0.32 M sucrose containing physostigmine or in 0.02 M NaH_2PO_4 with a Polytron homogenizer rather than a Potter–Elvehjem homogenizer. Sucrose homogenates were extracted with acetone and treated as described. Homogenates prepared in acid phosphate were heated for 10 min at 100°, centrifuged and the ACh-like activity in the supernatant fraction was measured by bioassay. Parallel bioassays showed a ratio of 1, irrespective of the homogenization medium (Table 2). Thus, as has been shown for brain [5], the biological activity in these extracts would appear to be due to ACh. This finding contrasts with the results shown in Table 1. The primary difference between the two sets of experiments was the homogenization technique.

A further observation suggests that sucrose homogenization contributes to the discrepancy in the parallel bioassays. In one experiment the sciatic nerves from a New Zealand rabbit were homogenized in 150 mM NaCl containing physostigmine and then extracted with acetone and treated as described. The ratio of the parallel bioassay was 1.17. In a parallel experiment, the nerves from two New Zealand rabbits were homogenized in 0.32 M sucrose and extracted in the same way. The ratios of the parallel bioassays were 1.66 and 1.63. The ACh in extracts prepared by both homogenization procedures was also measured by p.g.c. In each extract, the ACh-like activity measured on the guinea pig ileum was very nearly identical to that measured by p.g.c.: the ratio (ileum/p.g.c.) was 1.04 ± 0.14.

These results show that the additional ester(s) of choline in acetone extracts of sucrose homogenates of rabbit sciatic nerve depends upon the homogenization and extraction procedures. These findings may be explained in the following way. Potter–Elvehjem sucrose homogenates of sciatic nerve, in contrast to homogenates of brain tissue prepared in the same fashion, are poorly dispersed. When acetone is added, the particulate material, which contains choline acetyltransferase [6], aggregates to form a sticky mass. This aggregate likely inhibits the penetration of acetone and subsequent inactivation of enzymes that synthesize choline esters. In the presence of these enzymes, fatty acids other than acetate can be activated and converted to choline esters with greater nicotinic than muscarinic activities [5]. In contrast, NaCl homogenates prepared with a Potter–Elvehjem homogenizer, though poorly dispersed, do not form a sticky mass when acetone is added. This suggests that the clumping and likely inhibition of acetone penetration is due to adherence of sucrose to the particulate material, a phenomenon that was demonstrated for sucrose homogenates of brain tissue [7]. On the other hand, sucrose homogenates prepared with the Polytron homogenizer were finely dispersed. Although aggregation of particulate matter was observed when acetone was added to such homogenates, no discrepancy in the parallel bioassays was observed. Under these circumstances it would appear that there was minimal entrapment of cytoplasm by the particles formed upon homogenization and thus no impairment of the ability of acetone to inactivate choline acetyltransferase.

In conclusion, both bioassay and p.g.c. on extracts in which the enzymatic activity is inhibited show that sciatic nerves of rabbits contain no choline ester other than ACh. This conclusion is in accord with other work showing that stimulation of peripheral autonomic [8, 9] and motor nerves [10] releases ACh and no other choline ester, as shown by p.g.c.

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