Acetylcholine: Release from Neural Tissue and Identification by Pyrolysis–Gas Chromatography

Abstract. *Gas chromatographic analysis showed acetylcholine to be the only choline ester released from innervated longitudinal muscle of the guinea pig ileum by electrical stimulation. The amount of acetylcholine measured by gas chromatography agreed almost exactly with that measured by bioassay. Denervated longitudinal muscle produced no acetylcholine, and treatment of the muscle with tetrodotoxin markedly reduced acetylcholine output. The method permits the recovery and quantitation of amounts of acetylcholine as low as 5 nanograms in 5 milliliters of tissue perfusates.*

Evidence that acetylcholine is the chemical agent released by stimulation of any peripheral parasympathetic nervous tissue rests on indirect experiments. Early studies (*1*, *2*) showed that the material released by nerve stimulation has the biological activity of a choline ester and is antagonized by drugs that antagonize choline esters. The material can be shown in tissue effluents only if collected in the presence of a substance that inhibits cholinesterase. It is also destroyed by alkaline hydrolysis. Experiments with isolated peripheral excitable tissues showed that radioactive choline is incorporated into material that is released by neural stimulation and migrates like acetylcholine on paper chromatography (*3*). None of these experiments rules out the release of other esters biologically similar to but chemically different from choline esters.

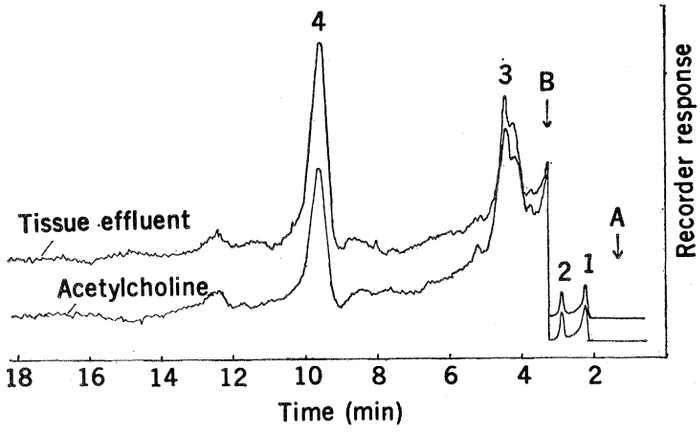
Table 1. Acetylcholine measured by bioassay and by gas chromatographic analysis of perfusates from the stimulated innervated longitudinal muscle strips of the guinea pig ileum. Values are given as nanograms of acetylcholine per milliliter of perfusate.

Experiment	Bioassay	Gas chromatographic analysis
1	52	53
2	111	114

The development in our laboratories (*4*) of a simple pyrolysis–gas chromatographic method that resolves and measures isomers and congeners of choline esters and other onium compounds provides a means of detecting the nature of the material released by stimulation of neural tissue and of quantifying this material.

The guinea pig ileum seemed an ideal tissue for this work since it contains cholinergic structures which are readily removed from the rest of the muscle (*5*). The procedure for preparing innervated and denervated longitudinal muscle of the ileum (*5*) was modified so that increased amounts of tissue were obtained. The entire ileum was stripped, and the longitudinal muscle was cut into 10 to 15 segments, 5 cm each, which were tied together in parallel.

The muscle bundle was then tied to a Phipps and Bird isotonic transducer in a bath containing 5 ml of Krebs solution to which was added physostigmine salicylate (1 μg/ml). Sufficient electrical stimulation was applied to produce maximum contraction. Perfusates collected from the resting and stimulated strips were adjusted to *p*H 4 to 5, and 10 μg of tetramethylammonium chloride was added as a coprecipitant; for quantitative studies 100 ng of butyrylcholine was added as an internal standard. The quater-



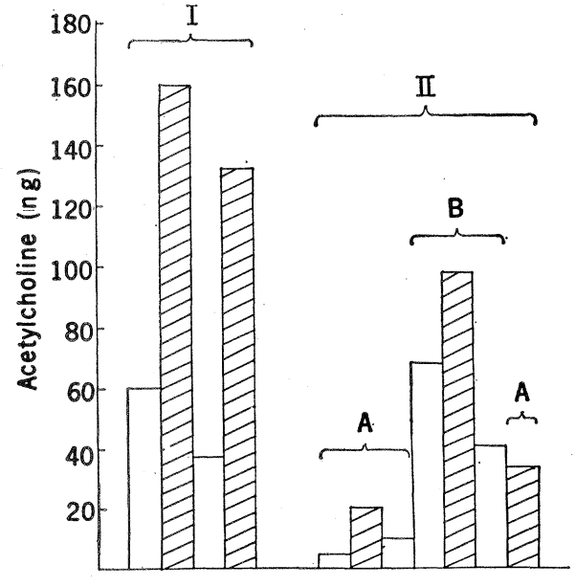


Fig. 1 (left). Chromatograms of a perfusate from stimulated tissue and of authentic acetylcholine after precipitation with potassium iodide–iodine and pyrolysis. At *A*, which indicates the moment of pyrolysis, the electrometer sensitivity was 6.4×10^{-10} amp. At *B* the sensitivity was increased to 6.4×10^{-12} amp. Peak 1, trimethylamine; peak 2, methyl iodide; peak 3, unidentified material arising as an artifact of the procedure; peak 4, dimethylaminoethyl acetate. No other peaks were observed as long as 90 minutes after pyrolysis. The tissue was stimulated for 30 minutes at a frequency of 10 per second at a duration of 0.2 msec. Fig. 2 (right). The release of acetylcholine from the innervated preparation of the ileum at rest and after stimulation (I). Clear bars represent the release at rest, the hatched bars, the release after stimulation. The perfusates were collected after 10 minutes, with 5-minute rest periods. The preparation was stimulated for 10 minutes (average weight, 420 mg) at a frequency of 10 per second and at a duration of 0.2 msec. Part II shows (*A*), the release of acetylcholine from the resting and stimulated preparation after incubation for 30 minutes with tetrodotoxin (1 μg/ml). The preparation was then washed repeatedly for 1 hour to remove tetrodotoxin (*B*). The tetrodotoxin was again added to the preparation (*A*). These data are the average of two experiments.

nary ammonium compounds were precipitated with a solution of potassium iodide–iodine, and the precipitates, after excess iodine was removed by sublimation, were dissolved in a minimum of acetonitrile and analyzed (*4*). Acetylcholine was measured (in amounts as low as 5 ng or about 0.03 nmole) from the calculated ratios of the electronically integrated peaks of the main products of pyrolysis which are, for acetylcholine, dimethylaminoethyl acetate, and for butyrylcholine, dimethylaminoethyl butyrate (*4*).

The peaks appearing from treatment of the perfusate of the stimulated innervated strip did not differ from those appearing after identical treatment of authentic acetylcholine, the major peak being dimethylaminoethyl acetate (peak 4, Fig. 1). After the perfusate was incubated with electric eel acetylcholinesterase, no such peak was detected. Quantitative assay of acetylcholine by the gas chromatographic method and by bioassay on the guinea pig ileum by two independent observers showed a striking similarity in the amounts of acetylcholine (Table 1).

This last observation not only demonstrated the validity of quantitation by this gas chromatographic technique but also showed that the biological activity of the acetylcholine-like material released by electrical stimulation can be attributed wholly to acetylcholine when assayed on the ileum. In a perfusate containing 500 ng of acetylcholine, no other choline ester was observed by gas chromatography.

Electrical stimulation more than doubled the amount of acetylcholine released (Fig. 2). Treatment of the preparation with tetrodotoxin (*6*) dramatically reduced the amount of acetylcholine released by the resting and stimulated preparation (Fig. 2). No acetylcholine was detected in perfusates of the denervated preparation even after prolonged electrical stimulation with a stimulus sufficient to produce maximum contraction of the denervated preparation. Thus, as indicated by bioassay (*2*), the acetylcholine released by stimulating the ileum arises from neural structures.

DENNIS E. SCHMIDT
PETER I. A. SZILAGYI
DANIEL L. ALKON
JACK PETER GREEN

Department of Pharmacology, Mount Sinai School of Medicine, New York 10029

References and Notes

1. V. P. Whittaker, *Handb. Exp. Pharmakol.* **15**, 1 (1963).
2. W. D. M. Paton and M. A. Zar, *J. Physiol. London* **194**, 13 (1968).
3. A. J. D. Friesen, J. W. Kemp, D. M. Woodbury, *J. Pharmacol. Exp. Ther.* **148**, 312 (1965); J. K. Saelens and W. R. Stoll, *ibid.* **147**, 336 (1965); M. B. Wallach, A. M. Goldberg, F. E. Shideman, *Int. J. Neuropharmacol.* **6**, 317 (1967).
4. P. I. A. Szilagyi, D. E. Schmidt, J. P. Green, *Anal. Chem.* **40**, 2009 (1968); D. E. Schmidt, P. I. A. Szilagyi, J. P. Green, *J. Chromatogr. Sci.* **7**, 248 (1969).
5. H. P. Rang, *Brit. J. Pharmacol. Chemother.* **22**, 356 (1964).
6. M. C. Gershon, *ibid.* **29**, 259 (1967).
7. Supported by NIH grant GM-14278-02, NSF grant GB-6248, and NIH training grant 5T1 GM99 to the Department of Pharmacology, Cornell University Medical College, used for P.I.A.S. and D.L.A.

29 May 1969

Cytochrome P-420: Tubular Aggregates from Hepatic Microsomes

Abstract. *Aggregates were formed when clear supernatants from hepatic microsomes that had been treated with steapsin were desalted and concentrated. These aggregates contain large numbers of uniform tubular elements. These structures resemble microtubules seen in many cells but differ in their substructure. The aggregates were rich in cytochrome P-420. Unlike soluble cytochrome P-420, the cytochrome P-420 contained in the aggregates combines with drugs to give the characteristic difference spectra normally seen only with cytochrome P-450 contained in intact microsomes.*

Microsomal cytochrome P-450 is the active form of the hepatic terminal oxidase which functions in the oxidation of steroids and xenobiotics. Attempts to render cytochrome P-450 soluble have failed, but treatment of microsomes with detergents, snake venom, or steapsin releases a soluble inactive form of the hemoprotein, cytochrome P-420 (*1*, *2*). Drugs which react with the oxidative system involving cytochrome P-450 also combine with the hemoprotein to give characteristic difference spectra (*3*). Soluble cytochrome

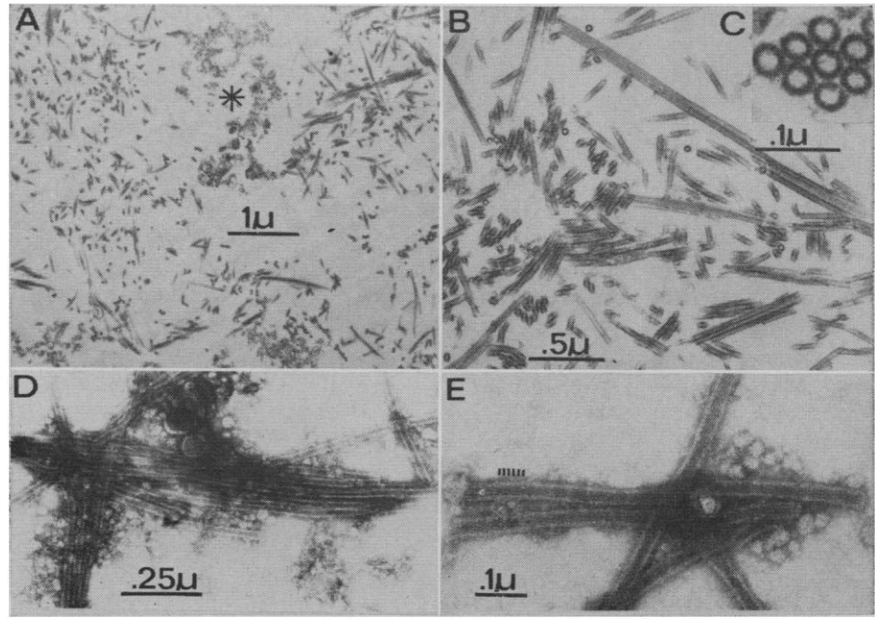


Fig. 1. Tubular elements from desalted concentrated supernatants of hepatic microsomes treated with steapsin. (A) Thin sections of fixed pellets reveal large numbers of tubules in clusters or scattered randomly in amorphous debris (*). (B) Higher magnification indicates the uniformity and rigidity of the tubular structures. Their similarity to microtubules is apparent. (C) Cross sections of the tubules reveal uniform circular profiles 340 to 360 Å in diameter. (D) Whole mounts of a suspension of tubules negatively stained with phosphotungstic acid. The rigid tubular structure is not disrupted by spreading and drying. (E) At higher magnification phosphotungstic acid outlines the substructure of the tubule wall. Hatch marks indicate parallel rows of globular subunits nearly perpendicular to the long axis of the tubule.