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.

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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

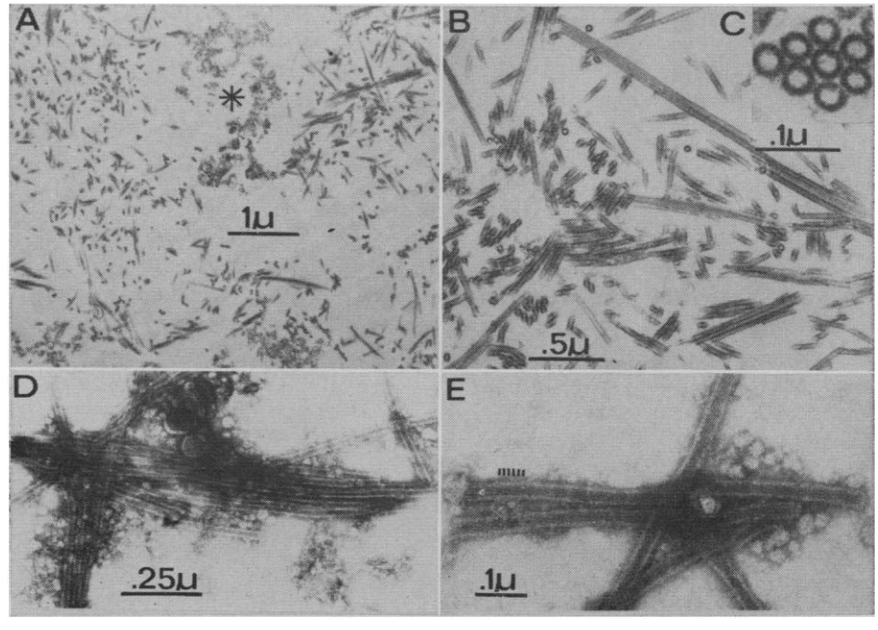

Fig. 1. Tubular elements from desalted concentrated supernatants of hepatic microsomes treated with steapsin. (A) Thin sections of fixed pellets reveal large numbers of tubules in clusters or scattered randomly in amorphous debris (*). (B) Higher magnification indicates the uniformity and rigidity of 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.

P-420 does not give these binding spectra with drugs. During attempts to purify cytochrome P-420, supernatants from steapsin-treated microsomes were desalted and concentrated before subjecting them to gel electrophoresis. The electrophoresis studies failed because desalting caused formation of aggregates that did not migrate on the gel. Aggregated material contained a large amount of cytochrome P-420. When drugs were added to suspensions of the material, binding spectra characteristic of those found when drugs are added to intact microsomes were seeen. This suggested the presence of macromolecules with special characteristics. The aggregated material consisted of large numbers of tubular particles of uniform structure.

Microsomes (100,000*g* pellet) prepared (*4*) from livers of male Holtzman rats (230 to 280 g) were incubated with steapsin (crude pancreatic lipase, Sigma grade II at a final concentration of 0.07 percent) under nitrogen for 24 hours at 4°C. The mixture was sedimented at 100,000*g* for 1 hour. The supernatant, which contained about 25 percent of the carbon monoxide–binding hemoprotein of the original microsomes, was desalted by passing it through a column of Sephadex G-25. The desalted supernatant, which was now slightly turbid, was concentrated by rotary evaporation (water bath at 40°C) to one-fourth volume and centrifuged for 1 hour at 100,000*g*. The pellet was fixed in a solution containing 1 percent osmic acid and 1 percent glutaraldehyde in Millonig's buffer (*5*) for 1.5 hours at 4°C. Fixed samples were washed in distilled water, dehydrated in alcohols, and embedded in Epon 812 (*6*). Thin sections were stained with uranyl acetate and lead citrate. Whole mounts were prepared by resuspending the pellets in distilled water and transferring small drops to carbon-stabilized Formvar-coated grids (*7*). Negative staining was carried out with 2 percent phosphotungstic acid at *p*H 4.

Pellets contained large numbers of uniform tubular elements randomly dispersed in amorphous debris, fat droplets, and membrane fragments (Fig. 1). Circular profiles were 340 to 360 Å in diameter with walls 65 Å thick and a clear central space 210 to 220 Å across. Except for their being slightly larger the tubules closely resembled microtubules found in abundance in a wide variety of cells (*8*). However, the substructure of the tubules observed in negatively stained whole mounts, also seen in Fig. 1, did not resemble that of microtubules prepared in a similar manner (*9*). Microtubules are composed of approximately 13 subfilaments in parallel association (*10*). The walls of the tubules examined in this study were composed of globular units arranged in rows almost perpendicular to the long axis of the tubules. A slight pitch in the parallel rows suggested that globular subunits might be joined in a continuous ascending spiral similar to the assembly proposed for tobacco mosaic virus (*11*).

The tubular elements were found in abundance in nine different preparations from rat liver. The entire sequence of preparation was examined to determine when tubules appeared. Pellets were obtained from the homogenate and microsomes before and after treatment with steapsin, and from the supernatants remaining after homogenization of liver, exposure to steapsin, and after desalting without concentration. Tubules were seen only in pellets obtained from desalted supernatants before or after concentration by evaporation. The procedure was repeated with deoxycholate (0.1 percent sodium deoxycholate at *p*H 7.4 in 1.15 percent KCl for 14 hours at 4°C) instead of steapsin. Tubules were not seen in the desalted concentrated supernatants. Adrenal microsomes and mitochondria, which contain abundant cytochrome P-450 (*12*), did not yield tubules when they were treated with steapsin and concentrated under the same conditions employed with hepatic microsomes, nor did membrane fractions obtained from blood platelets or erythrocytes.

The pellet from which the preparations shown in Fig. 1 was made contained 5.5 nmole of cytochrome P-420 per milligram of protein, as calculated from the molar extinction value of 111 cm^{-1} $mmole^{-1}$ given by Omura and Sato (*1*) for this hemoprotein. This concentration of cytochrome P-420 is about four times greater than that found in microsomes and about 90 percent of the most concentrated preparation made by Omura and Sato. The heme concentration as determined by a pyridine-hemochromogen method (*1*) was also found to be 5.5 nmole/mg, thus indicating that all of the heme was present in the form of cytochrome P-420.

The pellet containing the tubular elements was suspended in distilled water (1 mg of protein per milliliter) and divided into two 1-cm cuvettes. A zero base line was established with a Shimadzu model MPS-50 spectrophotometer, and the spectral change produced by the addition of sodium hexobarbital to one of the cuvettes (final concentration of 1.6 m*M*) was recorded. A typical type I difference spectrum (*3*) was observed with a peak at 385 nm and a trough at 431 nm. Aniline hydrochloride (final concentration of 16 m*M*) produced a typical type II difference spectrum (*3*) with a peak at 427 nm and a trough at 396 nm. The addition of KCl (final concentration of 0.3*M*) or urea (final concentration of 8*M*) clarified the suspensions and abolished both the type I and type II binding spectra. Binding occurred only when the tubular elements were present, and cytochrome P-420 must therefore be an integral part of the tubule or very closely associated with it. It is conceivable that the tubules may be the smallest units of hemoprotein complex that can function in the drug-metabolizing system.

The existence of a hemoprotein in tubular form is not without precedent. When hemoglobin from sickle cells is reduced, the molecules aggregate into structures resembling microtubules (*13*). The rigid polymers alter the shape of susceptible erythrocytes and result in the morbid clinical manifestations of the disease.

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