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Synthesis and radioenzymatic assay of an antimony analog of choline

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Since the *in situ* localization of acetylcholine (ACh) in nerve terminals and its site of release are still unclear, we have synthesized the stibonium analog of choline (Sb-choline) to be used as a specific marker for the localization of cholinergic compounds via combined X-ray microanalysis and electron microscopy. This compound is a substrate for choline kinase, choline acetyltransferase, and sodium-dependent uptake into rat brain synaptosomes, and it may be a useful tool for characterizing these processes biochemically (E. M. Meyer and J. R. Cooper, unpublished observations). In this communication, we describe the synthesis and isolation of these Sb-compounds, along with a sensitive assay for them that is based on phosphorylation by choline kinase with [^{32}P]ATP.

A 2.5 cm × 15 cm heavy wall Pyrex tube with a 1 cm o.d. neck for sealing was flushed with dry nitrogen and charged with 5 ml of bis-(2-methoxyethyl) ether ("diglyme" 6.2 g iodoethanol, and 6.0 g trimethylstibine (Ventron, Danvers, MA). The resulting solution was cooled to −70° under a nitrogen atmosphere before evacuating the tube to 0.05 mm pressure and sealing it. After slowly reaching room temperature, the tube was placed in a tube furnace and heated to 90 ±5° for 16 hr. (Caution! One attempted preparation in which the reaction mixture was heated to 150° for 1 hr resulted in an explosion.) After cooling, the tube contained colorless plates intermixed with a small amount of yellow solid under a clear, colorless liquid. The tube was cooled to −70° and opened, the solvent was decanted, and the crystals were then removed by dissolution in water. Lyophilization yielded 11.5 g (94.5 per cent of theory) of a white, hygroscopic powder.* An analytically pure sample was prepared by treatment of an aqueous solution of this material with sodium tetraphenylborate (TPB).The resulting white precipitate then was recrystallized from acetone to give colorless plates of the TPB salt, m.p. 201–203°.† This compound was identified by elemental analysis as $[(CH_3)_3SbCH_2CH_2OH]^+[(C_6H_5)_4B]^-$. The structure of this compound is shown in Fig. 1. Sb-choline was separated from a minor reaction product on a carboxylic acid cation exchange column (CG-50; Mallinkrodt; 0.4 × 5 cm) that was pre-washed with 0.1 M sodium acetate (pH 7.0). The Sb-choline was eluted with 5 ml of sodium acetate whereas the minor product [tentatively identified as $(CH_3)_3Sb(OH)_2$]‡ was eluted with 4 ml of 0.5 M HCl. This compound, presumably an Sb(V) species, forms a highly fluorescent product with the Morin reagent [1], whereas the Sb(III) Sb-choline does not. The recovery of Sb-choline from this column was over 90 per cent the purified Sb-choline was converted to the chloride on an anion exchange column (BioRad AGl-X8) for further work. Acetyl Sb-choline was synthesized by treating 10–50 mg of Sb-choline with 2 ml of acetic anhydride in the presence of 100 μl of pyridine for 30 min at 50°. This solution was lyophilized and redissolved in 5 mM sodium acetate (pH 4.0). Sb-ACh was separated from unreacted Sb-choline by high voltage paper electrophoresis (Fig. 2), eluting with 5 mM sodium acetate (pH 4.0) from the paper. Sb-choline and Sb-ACh were visually identified on paper with Dragendorffs reagent [1]. Between 65 and 84 per cent of the original Sb-choline was acetylated by this procedure.

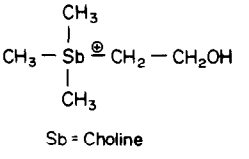

Fig. 1. Structure of the antimony analog of choline.

Labeled[122,124] Sb-choline was obtained by neutron activation (performed by Dr. Robert Litman, Lowell University, Lowell, MA). Neutron activation decomposed about 83 per cent of the original compound to several products, as indicated by high voltage electrophoresis (not shown). the remaining Sb-choline gave 7100 cpm/nmole 10 hr after activation, when counted in the combined $^3H/^{14}C/^{32}P$ window. Using these labeled compounds and [^{3}H]choline or [^{3}H]ACh, we found that stibonium analogs were separated from their ammonium counterparts by thin-layer chromatography, weak cation exchange columns, or descending paper chromatography, whereas the Sb-choline was separated from Sb-ACh by high voltage electrophoresis (Fig. 2). Thin-layer chromatography was performed on Eastman 13255 cellulose plates with a butanol–ethanol–acetic acid–water (8:2:1:3) system. This solvent system was also used with Whatman no. 3 paper for descending paper chromatography (separation time of 17 hr). Radioactivity was eluted from strips of paper or cellulose with 0.5 ml of 0.01 N HCl and collected in scintillation vials. For column chromatography, samples were prepared in 1 ml of 0.1 M sodium acetate buffer (pH 6.0) and put over CG-50 (0.4 × 5 cm) columns prewashed with this buffer until the effluent was pH 5.5 to 5.8. Radioactivity was eluted with successive 1-ml portions of sodium acetate buffer and then counted in 10 ml Aquasol (New England Nuclear Corp. Boston, MA). High voltage electrophoresis was performed as described previously [2].

Sb-choline was assayed according to a modification of the procedure of Goldberg and McCaman for choline [3], since Sb-choline is a substrate for choline kinase, although with a somewhat higher K_m (E. M. Meyer and J. R. Cooper, unpublished observations). Various quantities of Sb-choline were added to an incubation solution (500 μl) containing 1 mM [^{32}P]ATP (2.5 Ci/mole), 5 mM $MgCl_2$, 0.1 M sodium acetate buffer (pH 8.6), and 0.05 units of choline kinase (Sigma Chemical Co., St. Louis, MO). The choline kinase was preincubated for 10 min in the absence of radio-

* $[(CH_3)_3SbCH_2CH_2OH]^+I^-$. NMR ($D_2O$): δ 1.62 [br s, 11 H, $-Sb(CH_2)(CH_3)_3$] and 2.62 ppm downfield from DSS (t, J = 4.9 Hz, 2 H, $-CH_2-O-$). IR (Nujol): 3330 cm^{-1} (m); (KBr): 2995 (m), 2910 (m), 2865 (n), 1483 (w), 1392 (s), 1292 (m), 1253 (w), 1220 (w), and 1028 cm^{-1} (s).

† $[(CH_3)_3SbCH_2CH_2OH]^+[(C_6H_5)_4B]^-$. Anal. Calc. for $C_{29}H_{34}BOSb$: C, 66.58; H, 6.45. Found (Baron Consulting Co., Orange, CT); C, 65.39; H, 6.47.

‡ NMR (D_2O): δ 3.99 ppm (very br s). IR (Nujol): 3395 cm^{-1} (br, w); (KBr): 3210 (sh), 3120 (s), (m), 1397 (s), and 240 cm^{-1} (br, w).

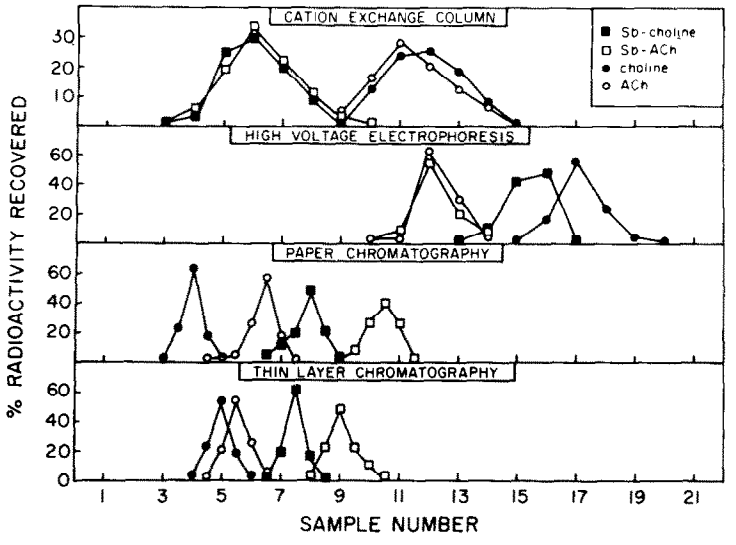

Fig. 2. Chromatographic properties of radioactively labeled choline, ACh, Sb-choline, and Sb-ACh. For the top panel, sample number (SN) refers to the number of millilitres eluted from the column; for the second and third panels, SN refers to the number of inches migrated from the origin; and in the fourth panel, SN refers to the number of centimetres migrated from the origin. See text for more details.

activity and Sb-choline to eliminate contaminating choline. The second incubation was terminated by cooling to 0°, and samples were chromatographed on anion exchange columns (AG1-X8; BioRad; 0.4 ×2.5 cm) to separate [^{32}P]ATP from labeled phosphoryl-Sb-choline [4]. Elution aliquots were counted, and the recovery from the column of phosphoryl-Sb-choline was found to be over 95 per cent (assuming 100 per cent phosphorylation of Sb-choline). Sb-ACh was assayed similarly before or after hydrolysis in 0.01 M NH_4OH for 30 min, followed by lyophilization and dissolution in the incubation medium. Losses due to extractions and counting efficiencies (about 88 per cent for ^{32}P) are accounted for in Fig. 3, which shows that Sb-choline was quantitatively phosphorylated by this assay procedure whereas Sb-ACh was a substrate for this assay only after hydrolysis. The lowest quantity of Sb-choline that we were able to assay in 0.5 ml of incubation medium was about 10 pmoles, which is comparable to the sensitivity reported for this procedure with choline by Goldberg and McCaman [3].

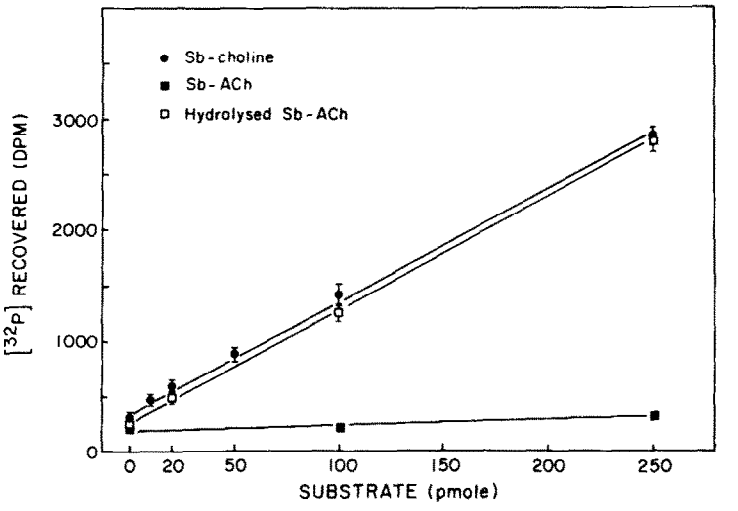

Fig. 3. Phosphorylation of Sb-choline or Sb-ACh by choline kinase. Sb-choline, Sb-ACh, or hydrolyzed Sb-ACh were incubated with choline kinase and [^{32}P]ATP as described in the text. The recovery of phosphorylated stibonium analogs was then determined and expressed here as dpm at increasing substrate concentrations.

Sb-choline could also be assayed in lysed tissue extracts containing choline, by first separating these analogs on the cation exchange column described Fig. 2. The eluate fractions containing Sb-choline or choline were brought to pH 8.6 with a small volume of 1 N NaOH, and aliquots thereof were assayed radioenzymatically with the choline kinase method. Neither choline nor any other tissue constituents interfered with the assay of Sb-choline following this column separation (not shown). Further, this column step obviated the tetraphenylboron/heptanone extraction which McCaman and Stetzler [4] found necessary to reduce excessively high tissue blanks (yielding apparently high choline and ACh levels).

The synthesis described here for Sb-choline is similar to those described for analogs of choline in which the nitrogen is replaced by phosphorus [5] or arsenic [6]. The use of a sealed vessel and "diglyme" as solvent, however, hastened our synthesis by allowing us to use a higher reaction temperature than previously reported. Other chloride and iodide forms of Sb-choline are hygroscopic white powders that are apparently stable indefinitely at −20°.

Sb-choline and Sb-ACh should be useful not only as readily assayable cholinergic analogs for biochemical studies, but as excellent substrates for the X-ray microanalytical localization of Sb-choline and Sb-ACh in cell structures. X-ray microanalysis coupled to electron microscopy is a technique with tremendous potential for localizing and quantifying specific elements in small biological samples, e.g. lead [7] or calcium [8] containing compounds in synaptosomes [see Ref. 9 for a review]. Cholinergic nerve terminals are unique in that they have a high affinity uptake system for choline as well as storage vesicles for ACh. Since we have found that Sb-choline is taken up into synaptosomes in a Na-dependent manner in low concentrations, acetylated, and then released as Sb-ACh by depolarization with 60 mM potassium (E. M. Meyer and J. R. Cooper, unpulished observations), it should be possible to specifically identify structural components in cholinergic terminals and localize Sb-ACh in them. We are currently conducting experiments along these lines.

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Induction of deoxyribonucleic acid damage in HeLa S_3 cells by cytotoxic and antitumor sesquiterpene lactones

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Sesquiterpene lactones constitute one of the largest classes of antitumor and cytotoxic substances of plant origin [for review, see Refs. 1–3]. The biological activities of these compounds seem to be related to their alkylating properties [4–6], although their modes of action are still not established. Alkylation of sulfhydryl groups in enzymes has been postulated as the major mechanism by which sesquiterpene lactones exert their effects on cell growth [7, 8]. However, an alternative hypothesis that DNA is a target for these agents seems to be more relevant to biological and biochemical properties of sesquiterpene lactones [9 and references therein]. Recently we demonstrated that cytotoxic and antitumor sesquiterpene lactones preferentially inhibit DNA synthesis in HeLa cells, and this inhibition seems to play a role in the growth inhibitory action of the compounds studied [9]. This paper explores the possibility that inhibition of DNA synthesis and of cellular growth by sesquiterpene lactones may be a result of damage to DNA that alters DNA template properties.

Parthenolide was isolated from *Tanacetum vulgare* L., and 1,10-epoxyparthenolide was obtained by epoxidation of the former [9]. Alatolide and eupatoriopicin were provided by Prof. B. Drozdz from the Medical Academy, Poznan, Poland; vernolepin and elephantopin by Dr. A. T. Sneden from the University of Virginia, Charlottesville, VA, U.S.A.; and costunolide by Prof. R. W. Doskotch from the Ohio State University, Columbus, OH, U.S.A. HeLa S_3 cells were grown in suspension culture as described previously [10] and were at mid-log at the time of drug addition. DNA synthesis in subcellular systems from G_1/S