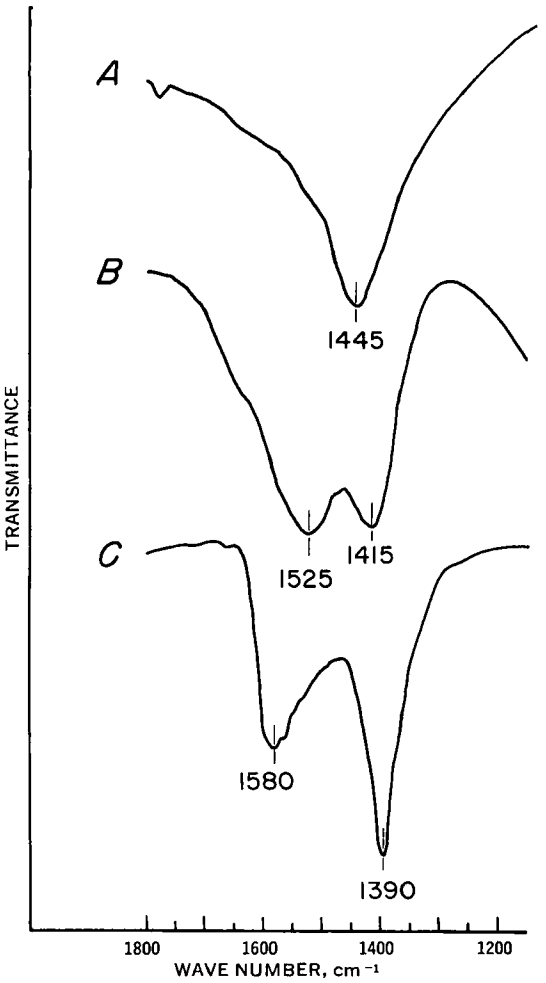

Figure 1—*IR spectra of 1200–1800-cm^{-1} region. Key: A, sodium carbonate; B, aluminum hydroxide gel; and C, sodium aluminum hydroxy carbonate.*

tion. Both methods clearly indicate that carbonate is associated with aluminum in aluminum hydroxide gel and should be recognized as an integral part of the structure of reactive aluminum hydroxide gel.

A report on the role of carbonate in aluminum hydroxide-type antacids is in preparation.

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Cytolytic Effects of Bromoacetylcholine on Neuroblastoma *In Vitro*

Keyphrases □ Bromoacetylcholine—cytolytic effects on neuroblastoma *in vitro* □ Neuroblastoma—cytolytic effects of bromoacetylcholine *in vitro* □ Cancer chemotherapeutic agents—cytolytic effects of bromoacetylcholine on neuroblastoma *in vitro* □ Cytolytic activity—bromoacetylcholine on neuroblastoma *in vitro*

To the Editor:

Neuroblastoma, probably the most common solid cancer in children (1), is treated with vincristine and cyclophosphamide. However, the remission rate in neuroblastoma patients is quite low and the duration of drug effectiveness is fairly short (2–4). Thus, new chemotherapeutic agents are needed to cope with this highly malignant tumor.

Recent studies revealed that the nature of neuroblastoma cells can be cholinergic, adrenergic, or inactive (5–8). It is also known that acetylcholinesterase is present in all cases and that these cells possess membranes that respond to acetylcholine and are electrically excitable, indicating the existence of cholinergic receptors at the membrane site (7, 9). Attempts made to destroy these tumor cells with an irreversible adrenolytic agent, 6-hydroxydopamine (10), have met with only partial success, possibly due to the inhibition of adrenergic components without an affect on the cholinergic parts of these tumor cells. Therefore, we proposed to kill these tumor cells with irreversible cholinolytics or with cholinergics such as bromoacetylcholine perchlorate, $BrCH_2COOCH_2CH_2{}^+N(CH_3)_3{\cdot}ClO_4{}^-$.

Bromoacetylcholine is a cholinergic agonist which stimulates both nicotinic and muscarinic receptors directly (11–13). However, when it is incubated with cell membranes for 15 min or longer, it binds irreversibly and specifically to the cholinergic receptor at the nicotinic site but not at the muscarinic site (11, 14). Therefore, it was thought that this compound might inhibit the growth of neuroblastoma cells. In this investigation, bromoacetylcholine inhibited the growth of neuroblastoma effectively at concentrations of 10^{-6}–10^{-5} *M*. Therefore, it is hoped that bromoacetylcholine can be used to treat the malignant tumor.

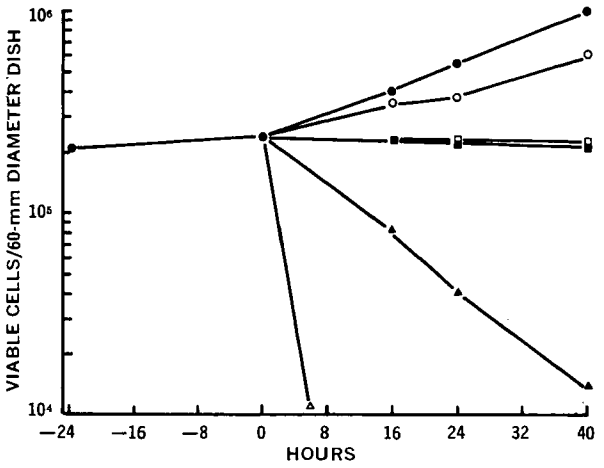

Figure 1—*Inhibition of neuroblastoma cells by bromoacetylcholine. Key:* ●, *control;* ○, *5* × 10^{-6} M *bromoacetylcholine;* □, *1* × 10^{-5} M *bromoacetylcholine for the first 16 hr and then washed away;* ■, *1* × 10^{-5} M *bromoacetylcholine;* ▲, *2* × 10^{-5} M *bromoacetylcholine; and* △, *1* × 10^{-3} M *bromoacetylcholine.*

Mouse neuroblastoma C-1300 clone N-18 was cultivated in modified Eagle medium[1] (containing, milligrams per liter: $CaCl_2$, 200; KCl, 400; $MgSO_4$, 97.72; NaCl, 6800; $NaH_2PO_4 \cdot H_2O$, 140; glucose, 1000; phenol red, 10; L-arginine hydrochloride, 126; L-cystine dihydrochloride, 31.29; L-glutamine, 292; L-histidine hydrochloride·H_2O, 42; L-isoleucine, 52.5; L-leucine, 52.4; L-lysine hydrochloride, 72.5; L-methionine, 15; L-phenylalanine, 32; L-threonine, 48; L-tryptophan, 10; L-tyrosine disodium salt, 52.1; L-valine, 46; calcium D-pantothenate, 1; choline chloride, 1; folic acid, 1; *i*-inositol, 2; nicotinamide, 1; pyridoxal, 1; riboflavin, 0.1; and thiamine hydrochloride, 1) supplemented with 10% fetal calf serum[2], 292 mg/liter L-glutamine, 2500 mg/liter $NaHCO_3$, 50 units/liter penicillin, and 50 units/liter streptomycin (7). The cells were grown at 37° under 95% air–5% CO_2 with 100% humidity.

For the culture experiments, 1 × 10^6 cells were placed in a Falcon tissue culture flask (75 cm^2) for 3–5 days until the cells were in the logarithmic phase of growth. They were then removed from the surface by a 15-min exposure to 0.05% trypsin in modified D_1 solution (7, 15). The cells were tapped off in fresh medium containing 10% fetal calf serum.

Aliquots of the cell suspension were incubated for 5 min with 0.04% trypan blue, and the titer of viable and nonviable cells was determined with a hemocytometer. Appropriate dilution was made, and the cell suspension was plated at 2 × 10^5 cells/dish (Falcon tissue culture dishes of 60 mm diameter) and grown for 24 hr. The medium was replaced with a fresh one as the control and with fresh medium plus various concentrations of bromoacetylcholine as the experimental samples. They were further incubated for 40 hr, and the cultures were removed at intervals for cell count.

[1] MEM, Earle's Powder, Catalog No. F-11, Grand Island Biological Co., Grand Island, N.Y.

[2] Catalog No. 614, Grand Island Biological Co., Grand Island, N.Y.

Figure 1 shows a complete inhibition of neuroblastoma cells by 1 × 10^{-5} *M* bromoacetylcholine with an ID_{50} of about 5 × 10^{-6} *M*. With higher concentrations of bromoacetylcholine (2 × 10^{-5}–1 × 10^{-3} *M*), neuroblastoma cells were destroyed and the cell number was reduced to below the original level. Figure 1 also shows an irreversible inhibition of neuroblastoma by bromoacetylcholine. The medium containing bromoacetylcholine was removed from dishes after 16 hr of incubation. The monolayers were rinsed and reincubated with fresh medium alone. No recovery of cell growth was observed with these treatments. These findings warrant further studies on using bromoacetylcholine to inhibit neuroblastoma *in vitro* and in experimental animals *(in vivo)*.

There are three important findings available regarding the pharmacology of bromoacetylcholine:

1. It produces cholinergic responses that can be blocked competitively by cholinergic blocking agents such as atropine (muscarinic) and tubocurarine (nicotinic) (11, 13).

2. It binds irreversibly to cholinergic receptors at nicotinic sites only but not at muscarinic sites (14).

3. It takes lower concentrations (10^{-5} *M*) to produce muscarinic responses but higher ones (10^{-3} *M*) to induce nicotinic responses (13, 14).

Based on these findings, we predict that when bromoacetylcholine is used to treat neuroblastoma patients, the muscarinic responses produced will be the major side effects. These can be eliminated by using atropine and its analogs along with bromoacetylcholine. Since the irreversible binding of bromoacetylcholine to cholinergic receptors at nicotinic sites is not affected by atropine, it will abolish side effects of bromoacetylcholine without reducing the ability of bromoacetylcholine to inhibit the neural tumor.

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Intestinal Bacterial Reduction of 4,4′-Dihydroxystilbene to 4,4′-Dihydroxybibenzyl

Keyphrases □ 4,4′-Dihydroxystilbene—intestinal bacterial reduction to 4,4′-dihydroxybibenzyl □ 4,4′-Dihydroxybibenzyl—intestinal bacterial reduction from 4,4′-dihydroxystilbene □ Metabolism—*trans*-stilbene, intestinal reduction of 4,4′-dihydroxystilbene

To the Editor:

We previously reported the metabolic reduction of the ethylenic double bond in *trans*-stilbene to yield 4,4′-dihydroxybibenzyl to the extent of 2.1% in rabbit urine and 10.6% in guinea pig urine (1, 2). Continued studies in our laboratories have shown that rats administered *trans*-α-^{14}C-stilbene intramuscularly in peanut oil excreted 4.1% of the dose as the bibenzyl compound in urine. These rat studies also have indicated that 40% of the original activity as compared to only 2.3% for rabbits is eliminated over 19 days in the feces. 4,4′-Dihydroxybibenzyl was detected by cochromatography with a reference compound in TLC Systems I and II[1]. It was the major metabolite in rat feces in a yield of 12.4% of the dose.

These species differences, which are consistent with reports (3, 4) that polarity and molecular weight determine the extent of biliary excretion in a given species, led us to postulate that biliary excretion and subsequent intestinal bacterial action are responsible for the reduction of the 4,4′-dihydroxy metabolite of *trans*-stilbene to 4,4′-dihydroxybibenzyl. That is, the increased yield of the bibenzyl compound in the rat could be due initially to an increased biliary excretion of either 4,4′-dihydroxystilbene *per se* or, more significantly, its glucuronide conjugate. It is of interest in this regard that enterohepatic circulation has been reported for the related compound, diethylstilbestrol, with extensive excretion into rat bile as the monoglucuronide followed by hydrolysis and reabsorption of diethylstilbestrol (5–7).

Recently, Scheline (8) also postulated that 4,4′-dihydroxybibenzyl is produced through reduction by intestinal microflora. His conclusion was based upon a decreased yield of the bibenzyl compound in urine of rats treated with the antibiotic neomycin sulfate or by bile duct ligation prior to the administration of *trans*-stilbene. Our studies confirm this hypothesis by direct incubation of dihydroxystilbene in an intestinal microflora extract.

Incubation studies were with 4,4′-dihydroxy-α-^{14}C-stilbene obtained by TLC (System I) of the phenolic fraction derived from an ether extract of enzyme[2]-hydrolyzed urine of rabbits administered *trans*-α-^{14}C-stilbene. The dihydroxy compound, which cochromatographed with reference material in TLC Systems I and II, was eluted from silica gel with methanol. This extract was concentrated, a few drops of polysorbate 80 were added, and the remaining methanol was removed before the sample was diluted to 1 ml with 0.1 *M* phosphate buffer (pH 7.4). An aliquot (0.1 ml = 4300 dpm) of the polysorbate 80 suspension was added to 1 ml of the incubation medium previously described (9, 10). The intestinal bacterial extract for this mixture was obtained from the intestinal and cecal contents of male Sprague–Dawley rats, 250–300 g. After incubation at 37° for 24 hr under nitrogen, the mixture was quenched with 1 *N* HCl (1 ml) and continuously extracted with ether for 48 hr. Controls were prepared in the same manner except that the intestinal bacterial extract was autoclaved for 17 min at 121° before addition to the sample tubes containing substrate.

Methanol solutions of the ether extracts of the control and test samples were investigated for transformations by TLC System I. Reference compounds cospotted with the methanol solution were visualized by quenching of the fluorescent TLC plates, while radioactive components were located using a radiochromatogram scanner[3]. Quantitation was by liquid scintillation counting of 0.64-cm (0.25-in.) strips from the TLC plates.

The TLC results from sample incubations indicated a yield of 14% of 4,4′-dihydroxybibenzyl at R_f 0.40, but there was no indication of the reduced product with TLC of the control incubation. Thus, these incubation studies confirm the reduction by intestinal microflora of 4,4′-dihydroxystilbene and, together with reports (9, 11) of the reduction of cinnamic acids to phenylpropionic acids, establish the conjugated ethylenic bond as among the moieties (12) reduced by intestinal microflora.

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[1] TLC systems employed were: I, toluene–piperidine (5:2); and II, benzene–methanol (9:1). TLC plates, 5 × 20 cm and 0.25-mm layer thickness, were precoated with silica gel F-254 (Brinkmann Instruments Co.).

[2] Hydrolysis was at 37° for 72 hr with 1000 units of β-glucuronidase–aryl sulfatase (Sigma Chemical Co.) per ml of urine.

[3] Packard radiochromatogram scanner model 7201, Packard Instrument Co.