Table IV—Contributions of Precorneal Factors in the Decrease of Pilocarpine Concentration in the Tear Film Following Topical Instillation of 25 μL of 1.00×10^{-2} M Pilocarpine Nitrate

Minutes	Productive Loss	Loss Due to Tear Turnover, μg/mL	Nonproductive Loss	Concentration in Precorneal Area
0	0	0	0	2083.5198
1	1.1258	43.5164	582.9035	1455.9741
2	2.7230	83.7475	1121.8004	875.2490
3	4.8423	113.5022	1520.3654	444.8103
4	7.4670	130.9236	1753.7251	191.4049
5	10.5208	139.1052	1863.3178	70.5768

instillation. Thus, it should be noted that although drainage is responsible for 42.7% of the total amount of drug instilled, it is the conjunctival absorption that is primarily responsible for the dramatic fall in the tear film pilocarpine concentration. The tear turnover is next in importance, contributing 6.9% of the decrease of concentration. Due to the rapid decline of drug concentration in the tear film, there is considerable loss of the driving force for productive drug absorption resulting in the poor bioavailability of topically administered drugs. Thus, decreasing the nonproductive loss of drug through the conjunctiva is as important a consideration as is circumventing the drainage loss in attempts to increase the bioavailability.

CONCLUSIONS

A model has been presented to account for the precorneal disposition of topically applied pilocarpine solutions. Due to the unknown nature of transcorneal penetration, the precorneal model was solved without making any assumptions about the mechanism of corneal transport. Experimentally obtained aqueous humor pilocarpine concentrations were used to obtain the rate of productive absorption. The rate constant for nonproductive loss, p_n, was found to be 8.84 μL/min, comparable with that used by Lee and Robinson (9).

Analysis of the model leads to a clearer understanding of the relative importance of the precorneal factors in reducing both the amount of drug in the precorneal area and the concentration of the drug in the tear film. It was shown that the conjunctival absorption is an important factor in reducing the amount of drug in the precorneal area and is the single-most important factor responsible for the reduction of drug concentration in the tear film and, hence, affects the driving force for productive absorption.

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Antineoplastic Activity of Tetrakis-μ-(trimethylamine-boranecarboxylato)-*bis*(trimethylamine-carboxyborane)dicopper(II) in Ehrlich Ascites Carcinoma

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Abstract □ A binuclear copper(II) complex derived from trimethylamine carboxyborane [tetrakis-μ-(trimethylamine-boranecarboxylato)-*bis*-(trimethylamine-carboxyborane)dicopper(II)] was shown to have antineoplastic activity in the Ehrlich ascites carcinoma screen. Metabolic studies demonstrated that the compound suppressed DNA and protein syntheses. The inhibition of DNA synthesis appeared to be due to reduction of the DNA polymerase activity and the regulatory enzymes of *de novo* purine synthesis. Preliminary data suggest that the compound is an initiation inhibitor of protein synthesis in Ehrlich ascites cells.

Keyphrases □ Copper(II) complexes—with trimethylamine carboxyborane, antineoplastic activity, Ehrlich ascites carcinoma screen □ Trimethylamine carboxyborane—complex with copper(II), antineoplastic activity, Ehrlich ascites carcinoma screen □ Antineoplastic agents—potential, copper(II)–trimethylamine carboxyborane complex, Ehrlich ascites carcinoma screen

Previously, series of trimethylamine cyanoborane and trimethylamine carboxyborane derivatives were reported to be active as antineoplastic agents against Ehrlich ascites carcinoma, Walker 256 carcino-sarcoma, P388 lymphocytic leukemia, B16 melanoma, and Lewis lung carcinoma growth (1, 2). Trimethylamine cyanoborane in Ehrlich ascites cells effectively inhibited DNA and protein syntheses as well as DNA polymerase and thymidylate synthetase activities. Scheller *et al.* (3) have demonstrated that amine carboxyboranes in dilute solutions bind to metal ions such as Zn^{2+} and Cu^{2+} as simple carboxylates and not as chelates. The synthesis and antineoplastic ac-

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Table I—Antineoplastic Activity of I Against Ehrlich Ascites Carcinoma Growth in Male CF_1 Mice [a]

Group	Survival on Day 10	Ascites Fluid, mL	Ascrit[b]	Inhibition, %
Control (0.05% polysorbate 80)				
Treated	6	7.17	39.3	—
10 mg/kg/d of I	5	0.04	18.5	99.7
20 mg/kg/d of I	6	0.32	34.0	96.2
6-Mercaptopurine	6	0.10	2.5	99.9

[a] $n = 6$. [b] Packed-cell volume.

tivity of a binuclear copper(II) complex derived from trimethylamine carboxyborane [tetrakis-μ-(trimethylamine-boranecarboxylato)-*bis*(trimethylamine-carboxyborane)dicopper(II) $[Cu_2(Me_3NBH_2CO_2)4.2Me_3NBH_2CO_2H]$, henceforth referred to as I] is reported herein.

EXPERIMENTAL

Preparation of Tetrakis-μ-(trimethylamine-boranecarboxylato)-*bis*(trimethylamine-carboxyborane)dicopper(II)—Cupric chloride ($CuCl_2 \cdot 2H_2O$) was purchased commercially[1]. Trimethylamine carboxyborane was prepared as described previously (1). Trimethylamine carboxyborane (1.8703 g, 15.9 mmol) was dissolved in 1 M NaOH (16 mL) and water (20 mL). Dropwise addition of 23 mL of a solution of $CuCl_2 \cdot 2H_2O$ (1.36 g, 8 mmol) in water (40 mL) produced a dark green solution which was allowed to stand overnight. Subsequent filtration through a fine fritted funnel removed a greenish brown sludge and left a dark green filtrate which was allowed to evaporate in the atmosphere. After 6 d, the solution had evaporated, leaving many small green crystals in a clear liquor. These crystals were removed by filtration and washed with chloroform (40°C); no $(CH_3)_3N \cdot BH_2COOH$ crystals were evident. The green crystals were then washed with a minimal amount of cold water to ensure removal of any trace of sodium chloride and dried *in vacuo*. The yield was 0.49 g (28%), mp 165°C (dec.); IR: ν_{BH} 2350 and $\nu_{C=O}$ 1665 cm^{-1}. The structure has been determined by single crystal X-ray analysis (4).

Anal.—Calc. for $C_{24}H_{68}B_6Cu_2N_6O_{12}$: C, 34.95; H, 8.31; N, 10.19. Found: C, 35.00; H, 8.49; N, 10.15.

Biological Studies—Male CF_1 mice (~30 g) were implanted intraperitoneally with 2×10^6 Ehrlich ascites tumor cells on day 0. A homogeneous suspension of I in 0.05% polysorbate 80–water was administered intraperitoneally at 10 and 20 mg/kg for 9 d to determine the inhibition of tumor growth. Mice were sacrificed on day 10, and the ascites fluid was collected from the peritoneal cavity. The volume and ascrit (packed-cell volume) were determined for each animal, and the present inhibition of tumor growth was calculated (5). For the metabolic studies, mice were treated on days 8 and 9 with 10 mg/kg ip of I. The animal was sacrificed on day 10, and the ascites fluid was harvested. The *in vitro* metabolic studies were performed at 0.25, 0.50, and 1 mM final concentrations of I.

In vitro incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine was determined using 10^6 Ehrlich ascites cells, 1 μCi of labeled precursor, minimum essential medium, and final concentrations of drug from 0.25 to 1.0 mM. The mixtures were incubated at 37°C 60 min and inactivated by trichloroacetic acid. The acid-insoluble labeled DNA was collected on glass filter disks[2]; RNA and proteins were precipitated on nitrocellulose filters by vacuum suction (6). Results are expressed as dpm of incorporated precursor/h/10^6 cells.

For *in vivo* studies, incorporation of thymidine into DNA was determined by the method of Chae *et al.* (7). One hour prior to the animal sacrifice on day 10, 10 μCi of [6-^{3}H]thymidine (21.5 Ci/mmol) was injected intraperitoneally. The DNA was isolated, and the tritium content was determined in a toluene-based scintillation fluid[3]. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard. Uridine incorporation into RNA was determined using 10 μCi of [5,6-^{3}H]uridine (22.4 Ci/mmol). RNA was extracted by the method of Wilson *et al.* (8). Using yeast RNA as a standard, the RNA content was assayed by the orcinol reaction. Leucine incorporation into protein was determined by the method of Sartorelli (9) using 10 μCi of [4,5-^{3}H]leucine (52.2 Ci/mmol). Protein content was determined by the Lowry *et al.* procedure (10) using bovine serum albumin as a standard.

In vitro and *in vivo* nuclear DNA polymerase activity was determined on isolated Ehrlich ascites cell nuclei (11). The incubation was that described by Sawada *et al.* (12) except that [methyl-^{3}H]deoxythymidine triphosphate (82.4 Ci/mmol) was used. The acid-insoluble nucleic acid was collected on filters[2] and counted. Nuclear RNA polymerase activities were determined on enzymes isolated from the nuclei. mRNA, rRNA, and tRNA polymerase enzymes were isolated using, respectively, 0.3 M, 0.04 M, and 0.0 M ammonium sulfate in magnesium chloride. The incubation medium was that of Anderson *et al.* (13) using [^{3}H]UTP (23.2 Ci/mmol). The acid-insoluble RNA was collected on nitrocellulose filters and counted[3].

Deoxythymidine as well as deoxythymidylate monophosphate and diphosphate kinase activities were measured spectrophotometrically at 340 nm at 20 min using reduced NAD (0.1 μmol) (14). Thymidine incorporation (21.5 Ci/mmol) into nucleotides was measured using the medium of Maley and Ochoa (14). The reaction medium was extracted with ether, and the aqueous layer was plated on cellulose[4] plates and eluted with 0.5 M formic acid–0.6 M LiCl (1:1). Areas which correlated with the R_f values of thymidylate monophosphate, diphosphate, and triphosphate standards were scraped and counted[3].

Carbamyl phosphate synthetase activity was determined using the reaction medium of Kalman *et al.* (15) in the presence of of ornithine and ornithine transcarbamylase; citrulline formed from ornithine was measured at 490 nm by the method of Archibald (16). Aspartate transcarbamylase activity was assayed using the incubation medium of Kalman *et al.* (15). The colorimetric determination of carbamyl aspartate was conducted by the procedure of Koritz and Cohen (17). Orotidine monophosphate decarboxylase activity was assayed by the method of Appel (18) using 0.1 μCi of [^{14}C]orotidine monophosphate (34.9 mCi/mmol); the $^{14}CO_2$ generated in 15 min was trapped in 1 M KOH[5] and counted. Thymidylate synthetase activity was determined using a postmitochondrial supernatant (9000 × *g* for 10 min) and 5 μCi of [5-^{3}H]dUMP (14 Ci/mmol) according to the method of Kampf *et al.* (19).

[^{14}C]Formate incorporation into purines was determined by the method of Spassova *et al.* (20) using 0.5 μCi of [^{14}C]formic acid (52.0

Table II—*In Vivo* Effects of I on Ehrlich Ascites Carcinoma of Male CF_1 Mice [a]

Biochemical Parameter	Control (0.05% Polysorbate 80), % of control	Compound I[b] % of control
[^{3}H]Thymidine incorporation into DNA	100 ± 8	43 ● 5
[^{3}H]Uridine incorporation into RNA	100 ± 9	116 ± 10
[^{3}H]Leucine incorporation into protein	100 ± 8	48 ± 6[c]
[^{14}C]Formate incorporation into purines	100 ± 12	59 ± 6[c]
Number of cells/mL of ascites fluid (×10^6)		
Enzyme activities	100 ± 9	45 ● 4[c]
DNA polymerase	100 ± 6	38 ± 5[c]
mRNA polymerase	100 ± 8	96 ± 9
rRNA polymerase	100 ± 9	96 ± 11
tRNA polymerase	100 ± 10	63 ± 7[c]
Ribonucleotide reductase	100 ± 6	101 ● 8
Phosphoribosyl pyrophosphate amidotransferase	100 ± 9	41 ± 3[c]
Inosinic acid dehydrogenase	100 ± 10	100 ± 9
Dihydrofolate reductase	100 ± 8	75 ± 6[c]
Carbamyl phosphate synthetase	100 ± 10	77 ± 8[c]
Aspartate transcarbamylase	100 ± 9	89 ± 8
Orotidine monophosphate decarboxylase	100 ± 10	106 ± 9
Thymidylate synthetase	100 ± 9	102 ± 9
Thymidylase levels		
monophosphate	100 ± 10	43 ± 6[c]
diphosphate	100 ± 12	89 ± 11
triphosphate	100 ± 9	90 ± 10

[a] Mean ± *SD*; $n = 6$. [b] Dosed at 10 mg/kg/d on days 8 and 9. [c] Significantly different from the control group, $p \leq 0.001$.

[1] Allied Chemicals, Morristown, N.J.
[2] GF/F.
[3] Fisher Scintiverse.
[4] PEI cellulose F.
[5] Hyamine Hydroxide.

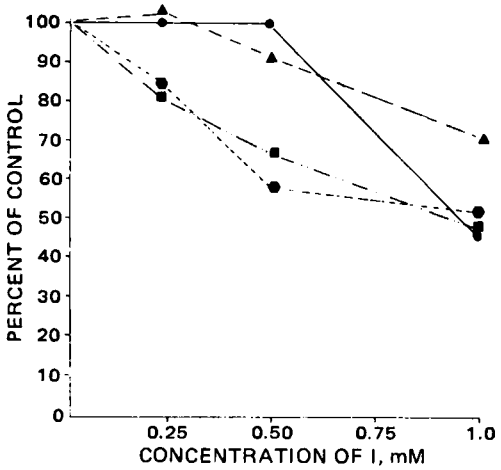

Figure 1—In vitro *effects of I on the incorporation of radiolabeled precursors into DNA, RNA, proteins, and purines of Ehrlich ascites cells. Key: (●) [^{3}H]thymidine incorporation into DNA; (▲) [^{3}H]uridine incorporation into RNA; (■) [^{3}H]leucine incorporation into proteins; (●) [^{14}C]formate incorporation into purines.*

mCi/mmol). Purines were separated on silica gel TLC plates eluted with *n*-butyl alcohol–acetic acid–water (4:1:5). After identifying R_f values consistent with the adenine and guanine standards, the plates were scraped and the radioactive content determined. Phosphoribosyl-1-pyrophosphate amidotransferase activity was determined on a supernatant fraction (600 × *g* for 10 min) measuring the reduction of 0.6 μmol of NAD at 340 nm for 30 min (21). Inosinic acid dehydrogenase activity was determined by the method of Becker and Löhr (22) using [8-^{14}C]IMP (61 mCi/mmol) and a 7000 × *g* supernatant. A sample of the reaction medium was plated on cellulose plastic-precoated TLC plates[4] and eluted with 0.5 M NH_4SO_4. The spot at the R_f value for xanthine monophosphate was scraped and counted. Dihydrofolate reductase activity was determined at 340 nm for 30 min as the oxidation of reduced NADP (23). Ribonucleotide reductase activity was determined by the method of Moore and Hurlbert (24) using [5-^{3}H]CDP (25 Ci/mmol). Ribose and deoxyribose nucleotides were separated on cellulose plastic-precoated TLC plates[4] eluted with 4% boric acid–4 M LiCl (4:3) and scraped at the R_f values consistent with the standard dCDP.

An *in vitro* method (25) was used to determine if I was an initiation or elongation inhibitor of Ehrlich ascites lysate protein synthesis by comparing with known inhibitors, pyrocatechol violet and emetine, using 1 μCi of [^{3}H]leucine (24.7 Ci/mmol). The reaction medium was spotted on filter paper[6] disks which, after drying, were treated for 10 min in boiling 5% trichloroacetic acid, for 10 min in cold 5% trichloroacetic acid, and washed with cold 5% trichloroacetic acid, ether–ethanol (4:1), and ether. The disks were dried and counted[3].

RESULTS

Compound I effectively inhibited Ehrlich ascites carcinoma growth at 10 and 20 mg/kg/d. The 10-mg/kg dose afforded greater inhibition of growth, *i.e.*, 99.7%, than the 20-mg/kg dose (Table I).

For the *in vivo* incorporation studies (Table II) the control values for thymidine incorporation into DNA for 60 min for day 10 Ehrlich ascites cells was 107,533 dpm/mg of isolated DNA, which was inhibited 57% by I. For uridine incorporation, the control was 51,193 dpm/mg of RNA isolated, which was unaffected by drug treatment. Leucine incorporation into protein for the control was 19,181 dpm/mg of isolated protein which was inhibited 52% by drug therapy. Formate incorporation into purine for the day 10 control was 28,786 dpm/mg of protein. *De novo* purine synthesis was inhibited 41% by I. It may be noted that drug administration for 2 d reduced the number of cells in the ascites fluid from 226 × 10^6 to 102 × 10^6 per milliliter.

Nuclear DNA polymerase activity for the control was 76,528 dpm/h/mg of nucleoprotein which was reduced 62% by administration of I. Nuclear mRNA polymerase activity for the control was 4867 dpm/h/mg of nucleoprotein, rRNA polymerase activity was 8751 dpm/h/mg of protein, and tRNA polymerase activity was 10,792 dpm/mg of protein. mRNA and rRNA polymerase activities were not affected by drug administration, but tRNA polymerase activity was suppressed 37%. Ribonucleotide reductase activity for the day 10 control was 153,791 dpm/mg of protein and was unaffected by drug administration.

[6] Whatman No. 3.

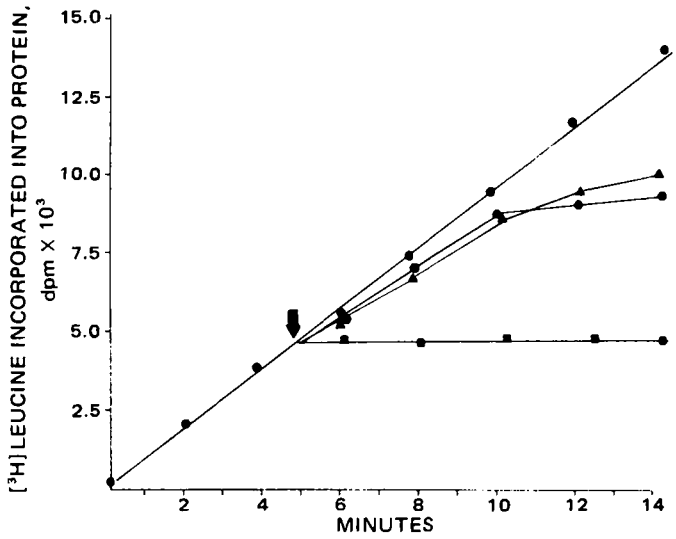

Figure 2—*Effects of I on the initiation and elongation of protein synthesis of Ehrlich ascites carcinoma cells. Key: (●) control; (●) pyrocatechol violet; (■) emetine; (▲) I; (⇓) drugs added at 100 μM concentrations.*

Phosphoribosyl pyrophosphate amidotransferase activity for the control resulted in a net change of 0.544 optical density units/h/mg of protein; drug administration for 2 d reduced the activity 59%. Inosinic acid dehydrogenase activity for the control was 36,530 dpm/mg of protein, which was essentially not affected by drug therapy. Dihydrofolate reductase activity for day 10 Ehrlich ascites cells was 0.514 optical density units/h/mg of protein; this was inhibited 25% by I. Carbamyl phosphate synthetase activity for the control was 0.128 mg of carbamyl phosphate formed/h/mg of protein, which was reduced 23% by I. Aspartate carbamyl transferase activity for the control was 7.526 mg of carbamyl aspartate formed/h/mg of protein, which was suppressed 11% by drug administration. Orotidine monophosphate decarboxylase activity for the control was 10,775 dpm of $^{14}CO_2$ generated in 15 min/mg of protein; this was not affected by drug administration. Thymidylate synthetase activity for the control was 103,328 dpm/mg of protein, which was not suppressed by I. [^{3}H]Thymidine incorporation into mono-, di-, and triphosphate pools were reduced 57, 19, and 10%, respectively. Deoxyribonuclease activity for the control was 247 μg of DNA hydrolyzed/h/mg of protein.

Preliminary whole cell *in vitro* incorporation studies demonstrated that I afforded an ID_{50} of 0.94 mM for the inhibition of DNA synthesis. For RNA synthesis the ID_{50} was 1.82 mM, and for the protein synthesis the ID_{50} was 0.863 mM. Using a supernatant fraction, the ID_{50} for formate incorporation into purine for I was 1.71 mM. Figure 1 demonstrates the *in vitro* effects of I on the incorporation studies for nucleic acid, protein, and purine biosyntheses.

DNA polymerase activity in isolated nuclei from Ehrlich ascites cells was inhibited significantly by I, with an ID_{50} of 0.454 mM. tRNA polymerase activity resulted in an ID_{50} of 0.433 mM. Phosphoribosyl pyrophosphate amidotransferase activity was also significantly inhibited by the copper complex with an ID_{50} of 0.523 mM. Deoxyribonuclease activity was inhibited significantly, with an ID_{50} of 0.682 mM. A number of other enzymes were not inhibited by I in the concentration range of 0.25–1.0 mM. These include carbamyl phosphate synthetase, aspartate carbamyl transferase, orotidine monophosphate decarboxylase, thymidylate synthetase, inosinic acid dehydrogenase, dihydrofolate reductase, ribonucleotide reductase, and mRNA and rRNA polymerase activities.

Figure 2 demonstrates that I does not immediately inhibit protein synthesis of Ehrlich ascites lysates, but rather there is a lag of several minutes before inhibition is observed. Protein synthesis inhibition by I resembles more closely the type of inhibition seen for pyrocatechol violet, an initiation inhibitor, rather than that by emetine, an elongation inhibitor of polypeptide synthesis.

DISCUSSION

The boron compounds were originally synthesized with the idea that they may act as antimetabolites of α-amino acids. They have been used

therapeutically in the past in neutron capture in cancer therapy. The parent compound, trimethylamine carboxyborane, at 20 mg/kg produced 82% inhibition of Ehrlich ascites growth. As can be seen, I is more potent, producing 96.2% inhibition at 20 mg/kg. Compound I significantly inhibited DNA and protein syntheses in Ehrlich ascites cells. The major sites in DNA synthesis that were inhibited include DNA polymerase and *de novo* purine syntheses. The regulatory enzyme of purine biosynthesis, phosphoribosyl pyrophosphate amidotransferase was significantly inhibited by I; the inhibition was of a magnitude to account for the observed inhibition of purine synthesis.

The trimethylamine cyanoboranes and carboxyboranes have been observed to inhibit DNA polymerase activity (1) in a similar manner as I. However, the former derivatives also significantly suppressed thymidylate synthetase activity. Compound I had no effect on thymidylate synthetase activity either *in vivo* or *in vitro*. It may be noted that heavy metals at low concentrations have been known to inhibit DNA polymerase activity by directly binding to the enzyme and form a stable complex with nucleotide triphosphates, polynucleotides, and DNA (26); however, in these studies the copper existed as an ion species. Further evidence indicates that metal ions are carcinogenic and mutagenic. The copper in I is securely bound to the molecule; thus, there is no reason to believe that it is in an ionic form.

Compound I moderately inhibited (23%) the regulatory enzyme of pyrimidine synthesis, *i.e.*, carbamyl phosphate synthetase. Moderate inhibition of dihydrofolate reductase by I was observed and may be important in one-carbon transfer in the synthesis of both purines and pyrimidines. Previous studies with trimethylamine cyanoboranes and carboxyboranes have shown that one-carbon transfer from *S*-adenosyl methionine was suppressed (1). Deoxyribonuclease activity was also suppressed, which indicates that I did not cause the release of hydrolytic enzymes from lysosomes and thus cause the degradation of nucleic acids.

The data for the protein experiment suggest that I is an initiation inhibitor of Ehrlich ascites protein synthesis. Compound I exhibited behavior similar to the initiation inhibitor pyrocatechol violet, which allows completion of the ongoing round of protein synthesis, rather than emetine (27), an elongation inhibitor which freezes the ribosome on the mRNA with an immediate cessation of polypeptide synthesis (28).

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