Penetration of Brain and Brain Tumor. VII. Tumor-Binding Sulfhydryl Boron Compounds[1,2]

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The discovery of a class of boron compounds, containing the sulfhydryl group, which become fixed to tumor tissue and to serum proteins is presented. A comparison is made between the toxicity and tissue-binding properties of $B_{12}H_{12}^{2-}$ and $B_{12}H_{11}SH^{2-}$. There are definite indications that SH groups on such anions are potent nucleophiles. Toxicity studies of this latter anion in rabbits are also discussed.

Failures were experienced[5] in treating brain tumor patients by boron neutron-capture irradiation[6] using *p*-carboxybenzeneboronic acid and sodium decahydrodecaborate. These compounds were extremely nontoxic,[7] both were freely diffusible, low-threshold substances, and levels attained in tumor, though appreciably greater than normal brain, were nevertheless lower than the concentration in blood. Such amounts in the vascular supply resulted in extensive radiation to the blood vessel walls and the subsequent impairment in their function.[5]

These results confirmed the need for boron compounds which would leave the vascular supply and become incorporated into tumor cells. This may appear to be a highly restrictive requirement, namely a tumor-binding compound. This is not the case, however, for high boron levels in organs far removed from the tumor site would in no way impair the utilization of this procedure, provided these tissues were not exposed to high thermal neutron fluxes.

The evaluation procedure of boron compounds in tumor-bearing animals was designed to determine which compounds were effectively bound to tumor cells.[8] This was accomplished by repetitive daily injections with a lapse of several days between the last injection of boron and sacrifice of the animals. In the course of screening a variety of boron compounds, a class of substances has been uncovered which shows the highly desirable property of being bound to tumor cells and yet with the attainment of low levels in blood. These compounds are boron hydride anions, which contain the sulfhydryl moiety.[9] They are dimercaptooctachlorodecaborate $(B_{10}Cl_8(SH)_2)^{2-}$ and mercaptoundecahydrododecaborate $(B_{12}H_{11}SH)^{2-}$. The former compound was administered as the sodium salt to C3H mice bearing a subcutaneously transplanted ependymoblastoma and, from the results shown in Table I, it is apparent that the boron levels in tumor were higher than those in blood, while invariably the concentrations in normal brain were extremely low and generally unmeasurable by the analytical technique used. This compound was quite toxic and at the doses injected an LD_{20}–LD_{50} was approached. Initially, the high toxicity of this compound was attributed in part to the appreciable percentages of chlorine in the molecule, since the dose administered was based upon an amount of boron per gram weight of animal. These findings prompted an evaluation of the mercaptoundecahydrododecaborate anion both as its cesium and sodium salts. The results are presented in Tables II and III. As with the dimercaptooctachlorodecaborate anion, the $B_{12}H_{11}SH^{2-}$ moiety achieved tumor:blood boron ratios which were appreciably greater than one, ranging from 1.4 to 20.0. Also low boron concentrations were found in normal brain and the magnitude of this differential between brain and tumor can be assessed by an examination of the ratios. In many instances, the boron level in

TABLE I

$Na_2B_{10}Cl_8(SH)_2$

μg of boron/g[a]		Ratio
Tumor	Blood	tumor:blood
3.3	1.8	1.8
3.6	1.9	1.9
2.9	1.0	2.9
0.9	<0.5	>1.8
13.1	1.2	10.9
4.0	0.8	5.0
9.3	<0.5	>18.6
4.7	<0.5	>9.4
5.9	1.3	4.5
6.8	1.4	4.9
2.5	0.8	3.1
3.7	0.9	4.1
5.8	1.2	4.8
2.0	1.2	1.7
7.3	1.6	4.6
6.6	<0.5	>13.2

[a] Total dose ranged from 140–175 μg of boron/g of mouse.

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(7) W. H. Sweet, A. H. Soloway, and R. L. Wright, *J. Pharmacol. Exptl. Therap.*, **137**, 263 (1962).

(8) D. S. Matteson, A. H. Soloway, D. W. Tomlinson, J. D. Campbell, and G. A. Nixon, *J. Med. Chem.*, **7**, 640 (1964).

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TABLE II

$Cs_2B_{12}H_{11}SH$

μg of boron/g[a]			Ratio	
Tumor	Blood	Brain	Tumor/blood	Tumor/brain
17.2	4.4	1.1	3.9	15.6
21.8	5.0	1.5	4.4	14.5
6.5	1.7	1.4	3.8	4.7
4.4	2.1	1.0	2.2	4.4
33.1	6.2	1.3	5.3	25.4
6.0	2.3	1.4	2.6	4.3
37.6	2.1	0.8	17.9	47.0
19.9	3.9	0.8	5.1	24.9
4.2	1.1	0.6	3.8	7.0
5.0	1.8	0.6	2.8	8.3
13.3	2.0	0.5	6.6	26.6
7.5	1.8	1.6	4.1	4.7
23.4	3.4	1.3	6.9	18.0
18.7	3.3	1.1	5.7	17.0
5.0	1.9	<0.5	2.6	>10.0
5.2	2.0	<0.5	2.6	>10.4
9.3	1.5	1.1	6.3	8.5
3.5	0.5	<0.5	7.0	> 7.0
5.6	0.5	<0.5	11.2	>11.2
7.2	1.6	<0.5	4.4	>14.4
30.3	2.8	<0.5	10.8	>60.6
5.9	1.9	<0.5	3.1	>11.8
5.0	1.2	<0.5	4.0	>10.0
			Av 5.5	

[a] Total dose ranged from 140–175 μg of boron/g of mouse.

TABLE III

$Na_2B_{12}H_{11}SH$

μg of boron/g[a]			Ratio	
Tumor	Blood	Brain	Tumor/blood	Tumor/brain
16.6	3.0	<0.5	5.5	>33.2
10.4	2.7	<0.5	3.9	>20.8
14.8	2.6	<0.5	5.7	>29.6
4.8	2.8	<0.5	1.7	> 9.6
24.7	5.8	<0.5	4.3	>49.4
18.9	2.2	1.4	8.6	13.5
61.5	7.2	1.4	8.6	44.0
51.5	3.5	1.2	14.7	42.9
40.0	2.8	2.0	14.3	20.0
4.8	3.4	0.6	1.4	8.0
10.4	1.0	1.0	10.4	10.4
9.8	1.1	0.6	8.9	16.3
5.5	1.2	1.0	4.4	5.2
20.0	1.0	<0.5	20.0	>40.0
			Av 8.0	

[a] Total dose ranged from 140–175 μg of boron/g of mouse.

normal brain was unmeasurable by the analytical procedure, even using the entire brain as a single sample.

The toxicity of these three sulfhydryl compounds were entirely comparable with one another and appreciably greater than the values observed for $B_{10}H_{10}^{2-}$ and $B_{12}H_{12}^{2-}$.[7] A comparison of the acute toxicity of the $B_{12}H_{12}^{2-}$ and $B_{12}H_{11}SH^{2-}$ are shown in the logarithmic probability plots in Figures 1 and 2. From the least-square regression equation,[10] the LD_{50} value was calculated for each anion. The dodecahydrododecaborate moiety had a value of 1025 ± 15 mg of boron/kg compared with only 73 ± 4 mg of boron/kg for $B_{12}H_{11}SH^{2-}$. There is no question that the incorporation of a sulfhydryl function into the boron hydride anions has a profound effect upon the compound's toxicological properties and it is highly possible that the biochemical mechanism responsible for the compound's incorporation into tumor contributes to this increase in toxicity. It seems probable that the sulfhydryl group on such an anion would be highly nucleophilic and in this manner be capable of interacting with electron-deficient sites in various biopoly-

(10) G. W. Snedecor, "Statistical Methods," 5th ed, The Iowa State University Press, Ames, Iowa, 1956, pp 123–125.

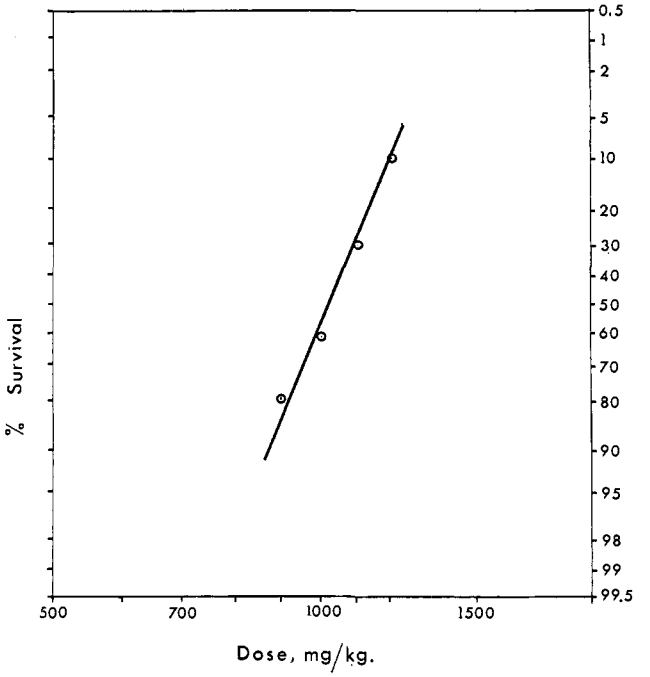

Figure 1.—Logarithmic probability plot of acute toxicity in CD1 male mice (Swiss Albino) by intraperitoneal injection. The LD_{50} was obtained by using the least-squares regression equation ($\hat{Y} - \bar{Y} = b(x - \bar{x})$; $\hat{Y} = 2.477x - 16.65$, where b, the regression coefficient, is 2.477). Calculated LD_{50} was 1025 ± 15 mg/kg.

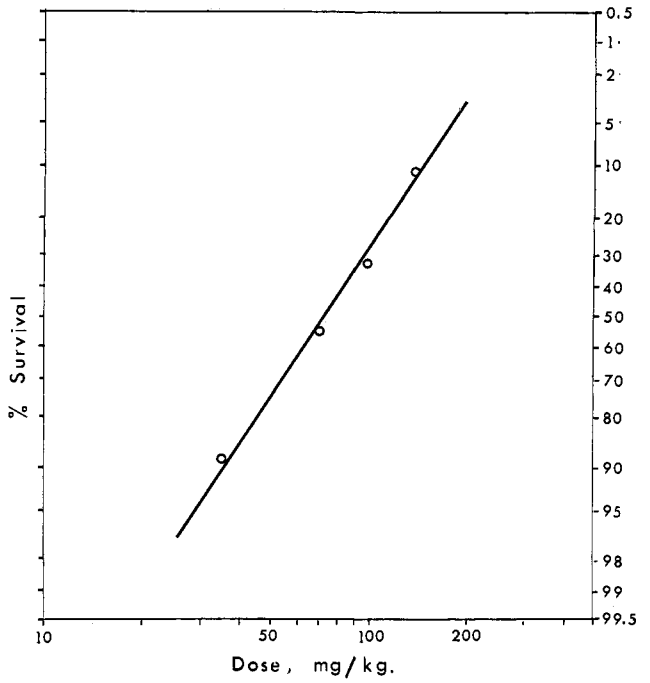

Figure 2.—Logarithmic probability plot of acute toxicity in CD1 mice (Swiss Albino) by intraperitoneal injection. The LD_{50} was obtained by using the least-squares regression equation ($\hat{Y} - \bar{Y} = b(x - \bar{x})$; $\hat{Y} = 0.557x - 1.889$, where b, the regression coefficient, is 0.557). Calculated LD_{50} was 73 ± 4 mg/kg.

mers forming a stable covalent linkage between the boron cage anion and the macromolecule. This hypothesis would account for the observed biological differences between $B_{12}H_{12}^{2-}$ and $B_{12}H_{11}SH^{2-}$. The former is extremely nontoxic, behaving as a low-threshold substance,[7] whereas the latter is more than ten times as toxic, being fixed to tissue.

While there is no positive correlation between the ability of serum proteins to bind chemicals and the localization of these substances within other tissues, nevertheless, a study of the possible differences in the attachment of these two anions to bovine serum albumin (BSA) may give some indications as to the validity of the above hypothesis. From the studies performed it is apparent that both anions are bound strongly to BSA under physiological conditions and even extensive dialysis failed to break this linkage. Precipitation of this boron-containing protein by trichloroacetic acid was likewise ineffective in splitting the boron–protein linkage. However, by use of ion-exchange chromatography the $B_{12}H_{12}^{2-}$ anion was totally removed from the protein by an anion-type resin, whereas the $B_{12}H_{11}SH^{2-}$ was largely unaffected and migrated with its protein component. Thus, it would appear that there is a difference between the binding of these anions to BSA. In the case of $B_{12}H_{12}^{2-}$ the linkage is completely a salt bond, whereas the $B_{12}H_{11}SH^{2-}$ undoubtedly is attached to the protein molecule through the formation of a covalent linkage. In this manner 4.5 moles of $B_{12}H_{11}SH^{2-}$ were attached to each mole of BSA. It is not possible at this time from the present experimental data to state whether the mode of attachment is *via* the formation of stable disulfide linkages between the boron cage and BSA, but certainly this is a distinct possibility.

The attractive tumor-binding properties of these sulfhydryl boron hydride anions resulted in the complete pharmacological testing of $B_{12}H_{11}SH^{2-}$ in rabbits prior to its evaluation in terminal cancer patients. The mode of administration was analogous to the procedure which would be used in man. Daily intravenous injections were performed on 5 successive days and the animal was then observed daily over a 30-day period following the last administration. The animal's physiological status was observed prior to and following drug injection, noting appetite, general appearance, neurological condition, rectal temperature, blood cell counts, blood coagulation time, urinalysis, blood urea nitrogen, total serum protein, albumin/globulin ratio, BSP excretion test, thymol turbidity test, serum electrolytes, serum creatinine, and serum glutamic oxaloacetic transaminase. These observations were compared as well with a control group of rabbits. From the studies it became apparent that rapid intravenous injection of isotonic solutions containing 13.5 mg of boron/ml of this anion were hazardous, being capable of provoking thrombosis, vasospasm, or exerting some type of phlebotoxic effect. This was revealed in autopsy findings on animals who succumbed from this procedure. There was evidence of pulmonary infarction, cerebral arterial thrombosis, multiple hemorrhagic infarction of the brain stem, and scar-like foci in the lungs, kidneys, liver, and intestinal tract. In animals, however, subjected to slow intravenous injection of more dilute isotonic solutions (6–7 mg of boron/ml) no apparent alteration in normal function was observed. Total doses of 200 mg of boron/kg over the 5-day span were well tolerated and following the 30-day observation period, these animals were sacrificed and a complete histopathological examination of the organs revealed no atropy or other abnormal pathology.

Analysis of tissue for boron content did show appreciable concentrations in liver, kidney, spleen, and adrenal with lesser amounts in skull, thyroid, and intestine, indicating once again fixation to tissue, but with low levels in blood comparable to those in brain. The levels of boron in hypophysis were also determined, since this tissue is partly of neural origin but without the normal blood–brain barrier and in this respect may be expected to simulate more closely brain tumor than other normal tissues do. The results of this pituitary study comparing boron concentrations in the hypophysis with those in blood and brain are presented in Table IV. It should be noted that pituitary/blood ratios ranged from 5.2 to 15.6.

TABLE IV

$Na_2B_{12}H_{11}SH$

	μg of boron/g[a]			
Rabbit no.	Pituitary	Blood	Normal brain	Ratio pituitary/blood
4	...	3.0	1.4	...
5	23.5	1.9	1.8	12.4
6	27.1	4.8	1.6	5.6
7	23.8	3.0	1.2	7.9
8	28.1	1.8	1.5	15.6
12	13.4	2.6	1.0	5.2

[a] Total dose was 200 mg of boron/g of rabbit.

The preparation of the sulfhydryl compound with reproducible biological properties has posed a problem, raising questions regarding the impurities produced in the synthetic sequence and stability of starting materials and products. Such investigations are currently underway.

Experimental Section

C3H mice bearing subcutaneously transplanted ependymoblastomas were used for the purpose of determining the tissue localization of the boron hydride anions $B_{10}Cl_5(SH)_2^{2-}$ and $B_{12}H_{11}SH^{2-}$. In order to obtain a more constant elevated blood level for a longer period of time than would be observed following a single injection, a dose of 35 μg of boron/g weight was administered daily for 4 or 5 days. The injections were then discontinued and the animals were sacrificed by ether inhalation 2 to 3 days following the last injection. The purpose of this scheme was to allow the compound to be cleared from the blood stream permitting a determination of tissue localization. The various tissues were removed, weighed or volumed, and analyzed for boron content.[11]

In the *in vitro* preparation of boron-labeled proteins, a typical synthesis was as follows. To a stirred, cooled solution of 100 mg of BSA in 15 ml of isotonic saline, 100 mg of $Cs_2B_{12}H_{11}SH$ was added. The mixture was stirred for 30–60 min, during which time complete solution occurred. The preparation was stored in the refrigerator overnight and then dialyzed. The dialyzed solution was used for further experiments. With the $B_{12}H_{12}^{2-}$ anion, some denaturation occurred upon its addition to the protein solution as noted by the formation of a small amount of precipitate. In this case, therefore, only the supernatant was used in chromatography.

(11) A. H. Soloway and J. R. Messer, *Anal. Chem.*, **36**, 433 (1964).

For chromatographic purposes, two 10-ml portions of a strong anion-exchange resin (amberlite IRA 400) were each washed with 100 ml of 1 N carbonate-free NaOH to be certain the resin was in the OH form of the cycle and then with 200 ml of water until the washes were neutral. Two-milliliter volumes (containing approximately 15 mg of protein with attached boron hydride anions) were passed through the resin columns, and volumes of 25–100 ml were collected. Aliquots were analyzed for boron content.

Acute toxicity studies in CD1 male (Swiss Albino) mice were carried out by a physiological saline.

For rabbit toxicity studies, male and female New Zealand white rabbits weighing between 2.1 and 3.1 kg were used. The cesium salt of the $B_{12}H_{11}SH^{2-}$ anion was converted to its sodium salt by ion-exchange resins and an isotonic solution of this compound at pH 7.2 was sterilized by passage through a Millipore filter (pore size: 0.45 μ) prior to its injection into the ear vein of the rabbit. A dose of 40 mg of boron/kg was administered daily for 5 successive days and then the animals were observed for 30 days following the last injection.

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Maleamic Acids That Affect Plasma Cholesterol and Penicillin Excretion

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A number of maleamic acids have been prepared by the reaction of maleic anhydride with an appropriate amine. In general, the amines are of the di- and triphenyl-substituted alkyl type for which synthetic procedures are given. Some of these have two asymmetric centers and in most instances both of the racemates were obtained in pure form. Compounds were prepared to show the biological effect of (a) the degree of substitution on the amide nitrogen atom, (b) systematic variation of substituents on the carbon atom α to it, and (c) variation in the positions of the aryl groups on the alkyl chain of the amine. For comparison purposes, the fumaramic acid analog of one of the most active maleamic acids and the succinamic acid analog of another were prepared. The hypocholesterolemic activity in rats and/or the inhibitory effect on penicillin excretion in dogs is reported. Structure–activity relationships are developed and biological effects of the major structural variations are emphasized. One of the maleamic acids, N-[2,3-bis(*p*-chlorophenyl)-1-methylpropyl]maleamic acid (benzmalecene) has been the subject of clinical investigation.

The effect of a maleamic acid, benzmalecene,[2] on penicillin excretion, uric acid transport, and plasma cholesterol in the dog, plasma cholesterol in the rat, and on the *in vitro* synthesis of cholesterol from mevalonic acid in rat liver homogenates has been reported from these laboratories.[3,4]

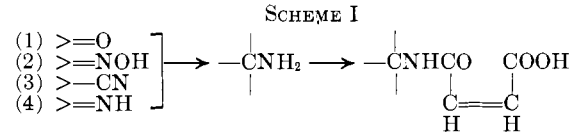
The action of benzmalecene on microbiological systems has been reported by Aaronson, *et al.*,[5] and by Holz, *et al.*[6] Inhibition of the active transport of bile acids across the intestinal mucosa by a number of maleamic acids has been studied by Lack and Weiner.[7]

Clinical evaluation in man has shown that benzmalecene has a hypocholesterolemic action,[8] inhibits the excretion of penicillin,[9] and has a uricosuric effect,[9,10] which, however, is not reflected in a decrease of blood uric acid levels.[9]

Benzmalecene (**1**, Table II) is a representative member of a series of related maleamic acids. The synthesis of other members of the series along with data for their inhibitory action on penicillin excretion in dogs and/or the hypocholesterolemic activity in rats comprise the subject matter of the present report.

All of the maleamic acids were prepared by the reaction of an amine with maleic anhydride. The appropriate amines were obtained from various precursors by procedures such as (1) the Leuckart reaction on an appropriate ketone, (2) catalytic hydrogenation of a corresponding ketoxime, (3) reduction of a nitrile, and (4) hydrogenation of a corresponding ketimine. These transformations are depicted diagramatically in Scheme I. The intermediate ketones, ketoximes

SCHEME I



(Table I), nitriles, and ketimines were prepared by standard procedures, the details of which are set forth in the Experimental Section. Known amines were pre-

(1) To whom inquiries should be addressed.

(2) Benzmalecene is the generic name for N-[2,3-bis(*p*-chlorophenyl)-1-methylpropyl]maleamic acid (α isomer): E. M. Schultz, J. B. Bicking, and V. D. Wiebelhaus, British Patent 901,438 (July 18, 1962).

(3) J. E. Baer, H. F. Russo, A. V. Brooks, and K. H. Beyer, *Pharmacologist*, **1**, No. 2, 53 (1959).

(4) J. W. Huff and J. L. Gilfillan, *Proc. Soc. Exptl. Biol. Med.*, **103**, 41 (1960).

(5) S. Aaronson, B. Bensky, and M. Shifrine, *ibid.*, **109**, 130 (1962); S. Aaronson, *J. Gen. Microbiol.*, **37**, 225 (1964); **39**, 367 (1965); *Nature*, **202**, 1355 (1964).

(6) G. G. Holz, Jr., L. Rasmussen, and E. Zeuthen, *Compt. Rend. Trav. Lab. Carlsberg*, **33**, 290 (1963).

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