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Whole-body irradiation of deuterated mice by the $^{10}B(n,\alpha)^{7}Li$ reaction

(neutron/boron/$^{2}H_2O$/radiation/therapy)

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ABSTRACT **Specific pathogen-free mice, 8–12 wk of age, were supplied with either acidified tap water or acidified 30 atom % $^{2}H_2O$ in tap water. Thirteen days later, when body water deuterium was about 20 atom %, mice were irradiated either by neutrons or by x-rays after intraperitoneal injection of boric acid. Mortality from whole-body neutron-boron radiation, unlike mortality from whole-body x-radiation, was not lowered by such deuteration. Time intervals to death of neutron-irradiated mice were compatible with the gastrointestinal syndrome. Neither species nor numbers of colonic bacteria were measurably altered by deuteration alone. Because the toxic, nonlethal range of deuterium substitution for aqueous hydrogen in mammals is approximately 1/5th to 1/3rd, these results indicate that partial deuteration of human tissues would improve neutron capture therapy of deep tumors. Neutron penetration would be enhanced and damage to normal tissues from photons would be decreased. The number of deuterium recoils due to neutron capture by hydrogen would also be decreased.**

Slow neutrons are much more reactive with ^{10}B than with the naturally occurring nuclides of mammalian tissue (1). Neutron therapy of boron-perfused tumors via the $^{10}B(n,\alpha)^{7}Li$ reaction (2) was proposed in 1936 (3). The tumoricidal potency of boron neutron capture therapy (BNCT) is demonstrable in small mammals (4–6). Clinical BNCT was initiated in 1951 to treat radioresistant brain tumors (7). Early trials were disappointing, in part, because inappropriate distribution of infused ^{10}B-enriched borates led to necrosis of normal brain structures (8). Current clinical trials of BNCT with the sulfhydryl borane $Na_2B_{12}H_{11}SH$ are encouraging (9).

The slow neutron dose to a deep tumor can be enhanced by increasing the energy of incident neutrons (10). Enhancement can also be achieved by partial deuteration of tissue water (ref. 11 and unpublished data), but such deuteration would be effective only if it did not lessen radiation damage by α and ^{7}Li particles. $^{2}H_2O$ is radioprotective for photon radiation (12–14). The present study indicates that $^{2}H_2O$, in common with other radioprotective agents (15), does not affect radiation damage by densely ionizing particles.

The range of isotopic substitution of deuterium for hydrogen in mammalian whole-body water that is toxic but not lethal is about 1/5th to 1/3rd (16). The experiments reported here assessed the effect of 1/5th deuteration of body water on the lethality of whole-body radiation from the neutron-boron reaction in Swiss albino mice of the Hale–Stoner strain (17). Deuterated and nondeuterated Hale–Stoner mice were also tested with whole body x-radiation. Mice of the CF-1, Simonsen Swiss albino, Füllinsdorf Swiss albino, and DBA/2 strains were used in previous photon radiation studies on the protective effect of $^{2}H_2O$ (12–14). The intent of our x-ray studies was to ascertain that Hale–Stoner mice are also amenable to protection from photon radiation by $^{2}H_2O$.

To supplement the present study, the effect of $^{2}H_2O$ alone on the colonic bacteria of nonirradiated mice was assessed quantitatively. It is known that colonic bacteria are important determinants of radiation lethality (18, 19). Conceivably, a significant alteration of colonic bacterial florae by ingestion of deuterated water could result in radioprotection without deuterium necessarily having a direct radioprotective effect on mammalian cells.

METHODS

Deuteration. Thirteen days before irradiation, 144–160 randomly bred 8- to 12-wk-old female, Hale–Stoner mice were distributed in small (28 × 18 × 13 cm) cages, 4 mice to a cage. Mice were bred and treated in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. Cages were supplied with 30 ± 1 atom % $^{2}H_2O$ in tap water (deuterated water) or with tap water (nondeuterated water). Water and nutritionally balanced food pellets (rodent laboratory chow no. 5001, Purina) were made available until 1–3 hr before irradiation. Deuterium and tritium levels of water were monitored by differential refractometry and liquid scintillation spectrometry, respectively. Our supplies of deuterated drinking water were contaminated by tritium at levels of <100 nCi/ml (1 Ci = 3.7×10^{10} Bq). For all experiments listed in Table 1 except R1A and R1P, traces of tritiated water were added to nondeuterated drinking water so that its tritium radioactivity would approximate that of the deuterated drinking water. In all experiments except R1A through R2P deuterated and nondeuterated drinking waters were sterilized and then acidified with 2 ml of concentrated HCl per liter prior to use. Mice were housed at ≈25°C and ≈55% relative humidity in a room illuminated 12 hr/day. One day before irradiation, cages with nondeuterated mice were renumbered so as to equalize the rank orders of mouse weight per cage in the deuterated and nondeuterated series of cages.

Irradiation. Irradiations were performed on 7 separate days spaced 1–2 months apart during an 11-month period of experimentation. Two radiation doses were used for similar experiments during the morning (lower dose, A) and afternoon (higher dose, P) of each irradiation day (Table 1). Except in experiment R1P, all neutron-irradiated mice received an intraperitoneal injection of sterile, 96 atom % ^{10}B-enriched boric acid dissolved in water, 24 μg of ^{10}B per g of body weight (bw), 10–25 min (mean ± SD, 17 ± 3 min) before the start of irradiation. In-

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Abbreviations: bw, body weight; RBE, relative biological effectiveness; BNCT, boron neutron capture therapy.

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Table 1. Dosimetry of whole-body radiation

Reactor experiments	^{10}B injected, μg/g of bw	Reactor power, kW	Duration of irradiation, sec	Anterior slow neutron fluence $\times 10^{12}$ cm^{-2}	Gamma dose, rads	Fast neutron dose, rads	Anterior ^{14}N(n,p)^{14}C dose, rads	Anterior ^{10}B(n,α)^{7}Li dose, rads	Effective dose Σ(kilorads × RBE)	
									Anterior	Posterior
R1A	24	100	450	0.92	37	18	20	179	0.8	0.2
R1P	5	500	450	4.61	188	90	101	203	1.3	0.6
R2A	24	200	300	1.23	50	24	27	239	1.0	0.3
R2P	24	200	450	1.85	75	36	41	359	1.6	0.4
R3A	24	200	450	1.85	75	36	41	359	1.6	0.4
R3P	24	200	600	2.45	100	48	54	476	2.1	0.6
R4A	24	200	450	1.85	75	36	41	359	1.6	0.4
R4P	24	200	600	2.45	100	48	54	476	2.1	0.6
R5A	24	200	450	1.85	75	36	41	359	1.6	0.4
R5P	24	200	600	2.45	100	48	54	476	2.1	0.6
X-ray experiments										
X1A	5	—	376	—	—	—	—	—	—	0.65
X1P	5	—	405	—	—	—	—	—	—	0.70
X2A	5	—	447	—	—	—	—	—	—	0.75
X2P	5	—	477	—	—	—	—	—	—	0.80

Each experiment involved 18 or 20 cages of mice, 4 mice per cage. Of those, half were deuterated and half were nondeuterated. On each day of irradiation, the lower and higher of two doses were used in the morning (A) and afternoon (P), respectively. RBE of the radiation doses in columns 6, 7, 8, and 9 are 1.0, 2.0, 2.0, and 3.7, respectively.

jection volumes were 0.02 ml/g of bw. In experiments R1P and X1A through X2P, all mice received similar injections of sterile, unenriched boric acid, 25 μg of B per g of bw, corresponding to 5 μg of ^{10}B per g of bw.

Ten untreated mice were given intraperitoneal injections of boric acid equivalent to 25 μg of B per g of bw and were bled 17 min later. The average (mean ± SD) boron concentration in blood was 23 ± 3 μg/ml. Because 96%-enriched [^{10}B]boric acid was used for most reactor experiments, radiation doses listed in columns 9–11 of Table 1 are referable to a blood ^{10}B concentration of 22 μg/ml.

Neutron exposures (symbol R in Tables 1 and 2) were carried out at the Brookhaven National Laboratory Medical Research Reactor. Slow neutron fluences were measured by activation of bare gold foils weighing 8–15 mg each. Fast neutron doses from the reactor have been measured (20). Gamma radiation was measured by thermoluminescence dosimetry (21). Doses from the ^{14}N(n,p)^{14}C reaction were obtained from

$$\text{Dose (rads)} = 0.22 \times 10^{-10}\,\Phi,$$

in which Φ = integrated neutron flux density (fluence, cm^{-2}) and tissue nitrogen concentration was assumed to be 3.2% (20) (1 rad = 0.01 gray). Doses from the ^{10}B(n,α)^{7}Li reaction were obtained from

$$\text{Dose (rads)} = 8.83 \times 10^{-6}\,F\,\Phi,$$

in which F is the fraction by weight of ^{10}B in tissue (20). The effective dose for each constituent radiation was obtained by multiplying the absorbed dose by the appropriate relative biological effectiveness (RBE). Currently accepted estimates of RBE used for these calculations were: 3.7 for the ^{10}B(n,α)^{7}Li reaction, 2.0 for the ^{14}N(n,p)^{14}C reaction and fast neutrons, and 1.0 for photons (22). The average ratio of posterior to anterior neutron fluence measured in deuterated and nondeuterated mice at the level of their greatest girth was 0.2. The anterior and posterior effective doses (columns 10 and 11 of Table 1) are calculated from incident neutron fluences and 1/5th incident fluences, respectively. Gamma and fast neutron doses were essentially uniform in the mice. Calibration factors for evaluation of thermal neutron flux density were obtained by absolute counting of gold, using a calibrated source of graphite-moderated neutrons. Nominal thermal neutron, fast neutron, and gamma doses listed in Table 1 should be within ≈10% of their true values.

Mice were irradiated at a vertically oriented, square (25.4 × 25.4 cm) hydraulically shuttered neutron irradiation port of the reactor, faced with a thickly varnished bismuth plate. They were irradiated in groups of eight—four deuterated and four nondeuterated—from an equally ranked pair of small cages. Each mouse was confined to a 8.9 × 2.5 cm plastic ultracentrifuge tube (polyallomer no. 326823, Beckman) with a nose air hole cut in its base. The tail was retroflexed and taped closely to the "dorsal" exterior surface of the tube. Deuterated and nondeuterated mice were taped to alternate positions at the perimeter of a stiff 25.4 × 25.4 cm support paper to facilitate transfer of each group of eight animals to and from the reactor port and to maintain mice in position during the exposure. When the paper was taped to the irradiation port, the "ventral" surface of each tube was separated from the bismuth plate only by a narrow (<1 cm) irregular air space and by the support paper. Reactor power was lowered to 10 kW during the exchange of one group of eight mice for another between neutron exposures, a procedure which added <0.1 rad of gamma radiation to the total dose. The exposure of experimenters to radiation from this study was <100 millirem (1 rem = 0.01 sievert).

X-ray exposures (symbol X in Tables 1 and 2) were carried out with a radiotherapy unit (Maxitron Two-Fifty, General Electric, Schenectady, NY) that was arranged to irradiate small animals 60 cm below the point source of radiation. The Maxitron was operated at 250 kV peak, 30 mA, with added filtration of 1 mm of Al and 0.5 mm of Cu. Each mouse was confined to a separate sector of a circular, 23-cm diameter plastic exposure chamber which rotated slowly (about 5 rpm) during irradiation. Each x-ray exposure was done with a group of four deuterated and four control mice from an equally ranked pair of small cages. Radiation measurements were calibrated with a string electrometer (model 570, Victoreen Instrument, Cleveland, OH). Average incident dorsal surface doses are listed in column 11 of Table 1. Deviations of incident radiation doses from the average are <4%.

Table 2. Correlation of postirradiation survival with deuteration

Reactor experiments	Fraction of mice surviving 30 days		Yule's correlation coefficient Q	χ^2 correlation coefficient	Probability of direct (+) or inverse (−) correlation between deuteration and survival, %
	Deuterated	Nondeuterated			
R1A	36/36	36/36	—	—	—
R1P	16/36	18/36	−0.1	0.06	(−) 19
R2A	39/40	40/40	−1.0	0.00	(−) <1
R2P	22/40	31/40	−0.5	3.58	(−) 94
R3A	17/40	24/40	−0.3	1.80	(−) 82
R3P	8/40	7/40	+0.1	0.00	(+) <1
R4A	33/40	36/40	−0.3	0.42	(−) 48
R4P	9/40	21/40	−0.6	6.45	(−) 99
R5A	14/40	13/40	+0.1	0.00	(+) <1
R5P	4/36	2/36	+0.4	0.18	(+) 33
Total survival	198/388	228/388	−0.2	4.38	(−) 96
Percent mortality	49% (44–56%)	41% (35–47%)			
X-ray experiments					
X1A	40/40	39/40	+1.0	0.00	(+) <1
X1A	38/40	32/40	+0.7	2.86	(+) 91
X2A	37/40	8/40	+1.0	39.82	(+) >99
X2P	29/40	2/40	+1.0	35.60	(+) >99
Total survival	144/160	81/160	+0.8	57.55	(+) >99
Percent mortality	10% (6–16%)	49% (42–58%)			

Yule's nonparametric correlation coefficients are calculated from Eq. 1. The χ^2 correlation coefficients are calculated from Eq. 2. Mortality percentages in parentheses following the percent mortality show the 95% fiducial confidence limits determined from figure 4 of ref. 25.

Acidified tap water and food pellets were made available to all deuterated and nondeuterated mice ad libitum beginning 1–3 hr after irradiation. Cages were examined daily to remove dead mice for 30 days after irradiation. Mortality data of deuterated and nondeuterated mice during each 3-day interval of the 30-day observation period were compiled (Fig. 1).

Bacteriology. Bacterial florae in 21 ± 4 mm (mean ± SD) segments of the proximal ascending colons of deuterated and nondeuterated mice were counted. Female litter mates were caged individually at 8- to 12-wk of age and were given acidified tap water or acidified 30% ^2H_2O in tap water for 11, 13, 14, or 18 days; they then were killed under ether anesthesia. Because data from animals killed on different days were similar they were combined to compile Table 3. For the data labeled "Effluents" each colon segment was rinsed with 5 ml of 0.85% aqueous NaCl through its lumen. For the data labeled "Homogenates" the segment of ascending colon, including its liquid fecal content, was homogenized in a 1-ml ground-glass tissue homogenizer (Reacti-Ware, Pierce) and was suspended in 5 ml of 0.85% aqueous NaCl. Serial 1:10 to 1:100,000 dilutions of each 5-ml specimen were made with 0.9% aqueous NaCl. A 0.1-ml aliquot of each dilution was spread onto a 10-cm diameter culture plate. Blood agar and colistin nalidixic agar plates were incubated at 36.0 ± 0.2°C in air with 5% CO_2. MacConkey agar plates were incubated in air. Similarly inoculated blood agar, colistin nalidixic acid agar, and kanamycin/vancomycin agar plates were incubated anaerobically in a 2.4-liter sealed jar (Baltimore Biological Laboratory Microbiology Systems). Plates from each dilution were examined, and those that yielded 30–300 colonies were selected for counting and subsequent identification.

Statistics. Yule's correlation coefficient (23) is

$$Q = (y_1x_2 - x_1y_2)\,(y_1x_2 + x_1y_2)^{-1}, \qquad [1]$$

in which x_1 and y_1 are the numbers of deuterated mice that died and survived, respectively, and x_2 and y_2 are the numbers of nondeuterated mice that died and survived, respectively.

Yates' χ^2 coefficient (23, 24) is

$$\chi^2 = (x_1 + y_1 + x_2 + y_2)[|x_1y_2 - y_1x_2| - 0.5(x_1 + y_1 + x_2 + y_2)]^2\cdot[(x_1 + x_2)(y_1 + y_2)(x_1 + y_1)(x_2 + y_2)]^{-1}. \qquad [2]$$

RESULTS

The reactor experiments (Tables 1 and 2) demonstrated either an inverse or a weak, statistically negligible (R3P, R5A, and R5P: $Q \leq 0.4$; $\chi^2 \leq 0.18$) correlation between deuteration and postirradiation survival. In two of the four x-ray experiments (X2A and X2P in Tables 1 and 2), deuteration-survival correlations were highly significant ($\chi^2 \geq 39.8$) and strong ($Q = +1.0$). In the other two x-ray experiments (X1A and X1P), correlations were fairly strong ($Q \geq +0.7$) but statistically insignificant ($\chi^2 \leq 2.86$) because these doses (X1A, 650 rads; X1P, 700 rads) were insufficient for demonstration of the radioprotective effect.

Selective bacterial growth media (26, 27) yielded Enterobacteriaceae, Lactobacillaceae, Streptococcaceae, and Bacteroidaceae on MacConkey, colistin nalidixic acid, colistin nalidixic acid, and kanamycin/vancomycin agar plates, respectively. *Escherichia coli*, *Lactobacillus* species, enterococci, and two species of Bacteroidaceae were usually present. Although Bacteroidaceae appeared to be increased in number by deuteration when they were counted in colonic rinse effluents, their numbers proved to be approximately equal in deuterated and nondeuterated mice when they were counted in colonic tissue homogenates (Table 3).

DISCUSSION

Partial deuteration of mice by ingestion of 30% deuterated water for nearly 2 wk does not lessen the susceptibility of mice to lethal doses of whole body irradiation from the neutron-^{10}B reaction. Thus, ^2H_2O shares the general property of radioprotective chemicals of being ineffective against densely ionizing particles (15). The data also confirm previous studies that indicate that partially deuterated mice are afforded significant

Table 3. Colonic bacterial florae in mice

	Fraction of mice yielding species				$\log_{10}$ [colony-forming units per mm of ascending colon]			
	Effluents		Homogenates		Effluents		Homogenates	
	D	C	D	C	D	C	D	C
Enterobacteriaceae								
Escherichia coli	11/11	12/12	8/8	6/8	3.1 (2.5–3.5)	2.9 (2.4–3.8)	3.4 (2.5–4.2)	3.9 (3.3–4.9)
Klebsiella pneumoniae	5/11	5/12	1/8	2/8	2.5 (1.8–3.4)	2.5 (1.4–3.6)	2.3	2.6 (2.4–2.8)
Enterobacter cloacae	1/11	4/12	0/8	2/8	2.4	2.2 (1.7–2.7)	—	4.3 (3.7–4.9)
Enterobacter agglomerans	2/11	3/12	0/8	0/8	3.4 (3.3–3.5)	2.6 (2.0–3.3)	—	—
Proteus mirabilis	1/11	1/12	0/8	0/8	3.6	2.8	—	—
Proteus vulgaris	3/11	1/12	0/8	0/8	2.0 (1.3–2.5)	2.2	—	—
Lactobacillaceae								
Lactobacillus species	10/11	12/12	8/8	8/8	5.6 (4.2–6.6)	5.3 (3.8–6.7)	5.4 (4.6–5.9)	5.5 (4.2–6.1)
Streptococcaceae								
Enterococci group	11/11	11/12	4/8	4/8	5.1 (4.2–6.2)	4.9 (1.8–6.6)	4.9 (4.2–5.4)	5.2 (4.4–6.2)
Viridans group	3/11	7/12	0/8	0/8	6.0 (5.8–6.4)	5.3 (4.8–5.9)	—	—
Bacteroidaceae								
Bacteroides vulgatus	12/12	8/11	8/8	8/8	5.9 (4.3–6.7)	4.5 (3.6–5.5)	4.5 (3.6–5.5)	4.5 (3.6–5.3)
Bacteroides diastonis	11/12	11/11	8/8	8/8	6.4 (5.3–7.8)	5.4 (4.8–6.6)	4.5 (4.1–5.3)	4.6 (3.3–5.6)

Mice were nonirradiated and either deuterated (D) or nondeuterated (C). Entries in the last four columns on the right show the decadic logarithms ($\log_{10}$) of the mean and range of the number of colony-forming units of the bacterial species per mm of proximal ascending colon, measured in colonic rinse effluents and colonic homogenates. A fraction indicates the number of mice examined (denominator) that yielded a bacterial species (numerator).

protection against the lethality of whole-body photon radiation (12–14). These results suggest that moderate partial deuteration of body water would improve boron neutron capture irradiation therapy of malignant tumors because it would increase the penetration of neutrons to boron-perfused tumors without affecting lethal effects of short-range ^{7}Li and α particles from the neutron-^{10}B reaction. Moreover, partial deuteration of tissues would decrease the number of 1.3-keV recoil deuterons (28) and γ photons (A. M. Brues, cited in ref. 12) due to the $^{1}H(n,\gamma)^{2}H$ reaction.

When postirradiation survival data from the four x-ray experiments (X1A–X2P) were combined, χ^2 was 57.55, indicating a >99% probability of correlation between deuteration and postirradiation survival. Reactor data yielded $\chi^2 = 4.38$ and $Q = -0.2$, which indicate a minimal net enhancement of radiation lethality by deuteration. If deuteration physically decreased the integral whole-body radiation dose, either the decrease was negligible or was compensated by biological enhancement of radiation effects.

The bacteriological data of Table 3 suggest that radioprotective effects of $^{2}H_2O$ were not mediated by deuterium isotope effects on intestinal bacteria alone because few differences were observed in microflorae between deuterated and nondeuterated animals. The disparity in Enterobacteriaceae colony counts between effluents and homogenates might be an artifact caused, for example, by putative deuterium-induced inhibition of bacterial affinity to colonic epithelium. Bacterial culture methods used in this study were selected to isolate major species that exist in the normal mammalian colon and are also capable of identifying intestinal pathogens such as *Salmonella*, *Shigella*, and *Pseudomonas* species. Preliminary fecal cultures of mice that drank nonacidified water showed *Pseudomonas* species sporadically, but pseudomonads were never cultured from mice maintained on acidified water.

The histogram compiled from the combined mortality data of these experiments (Fig. 1) reflects the disparity between survival times of mice irradiated with comparably lethal doses of photon and particle radiation (29, 30). The distribution of deaths in cages was found to conform with binomial random distribution statistics (unpublished data). Most frequent survival times after lethal x-irradiation were between 10 and 12 days. However, comparably lethal exposure of boronated mice to the nuclear reactor most frequently resulted in much shorter survival times, 4–6 days. Such long and short postirradiation survival intervals have been attributed to radiation-induced inhibition of cell proliferation in bone marrow and in intestinal epithelium, respectively (31). Disproportionate neutron irradiation of the abdomen might explain short postirradiation survival times. However, boron neutron irradiation of mice under identical conditions, except that their backs rather than their

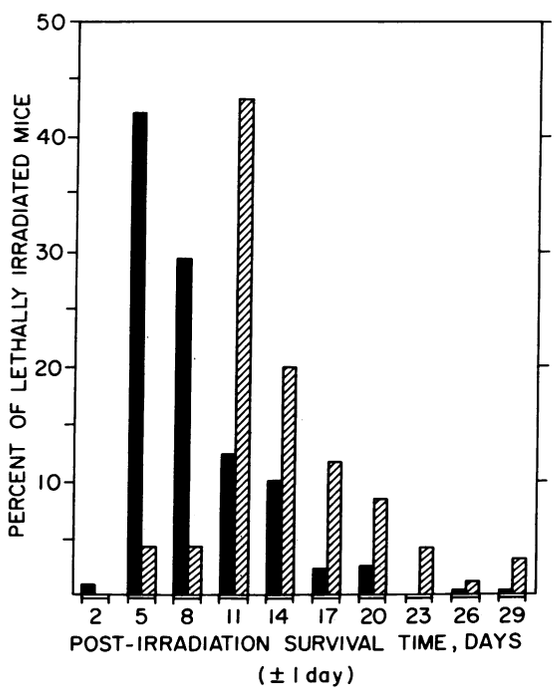

FIG. 1. Duration of survival of lethally irradiated mice. Solid bars show percentages of such animals dying within ten 3-day intervals after the day of $^{10}B(n,\alpha)^{7}Li$ irradiation. Adjacent diagonally lined bars show corresponding percentages after x-irradiation.

abdomens faced the reactor, most frequently resulted in the same 4- to 6-day postirradiation survival interval (unpublished data). The postirradiation survival intervals could result from more repair of (or more resistance to) damage of intestinal epithelial stem cells than of hematopoietic stem cells by photon, but not by particle, radiation.

Thirteen days after giving 30% deuterated drinking water to mice, the deuterium level in serum water approaches 20% (32). These experiments show that radiation lethality from the neutron-boron reaction is not decreased by such deuteration of body water. We conclude that the therapeutic effectiveness of BNCT of tumors may be improved by deuteration. Partial deuteration of the body water of patients during boron neutron capture irradiation would increase neutron penetration to the depth of a tumor and decrease the lethal effects of concomitant, poorly localized γ radiation on normal tissues.

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