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Preparation of Tumor Specific Boron Compounds

I. In Vitro Studies using Boron-labeled Antibodies  
and Elemental Boron as Neutron Targets

by

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Abstract

The diazonium salt of 1-(4-aminophenyl)-1,2-dicarba-closo-dodecaborane(12) has been incorporated into anti-bovine serum albumin and antibody proteins specific to human and mouse histocompatibility antigens. The incorporation of the boron-label (natural isotopic abundance) into the antibody is accomplished under sufficiently mild conditions so as to have minimal effect on the activity and specificity of the resulting boron-labeled antibody. The amount of boron incorporated

into the antibody is sufficient to cause specific cellular destruction when subjected to neutron radiation in vitro. It has also been demonstrated that particulate boron (natural isotopic abundance) is incorporated into Walker 256 cells which can be specifically destroyed with neutron radiation.

The significance of these results using boron-labeled antibodies and elemental boron strongly suggests that the concept of incorporating boron into proteins which exhibit marked tumor-specificity is of great potential for use in neutron-capture therapy.

The potential use of boron-containing compounds in cancer therapy is based on the unique nuclear property of the boron-10 nucleus to absorb thermal neutrons. This approach to cancer therapy is based on the liberation of high-energy fission fragments following neutron capture. The incident thermal neutrons have a relatively low energy (0.025 ev) which gives rise to a very high energy  $\alpha$ -particle (2.4 Mev) following capture by  $^{10}\text{B}$  and fission.

The essential factors for the selective destruction of tumors have been reviewed.<sup>2,3</sup> The major requirements for the successful appli-

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(2) A. H. Soloway, in "Progress in Boron Chemistry," A. L. McCloskey and H. Steinberg, Ed., Pergamon Press, Inc., New York, N.Y., 1964, pp. 203-234.

(3) G. L. Brownell, A. H. Soloway, and W. H. Sweet, "Boron Capture Therapy," in "Modern Trends in Radiotherapy," T. J. Deeley and C. A. P. Wood, Ed., Butterworths, Inc., London, 1967, pp. 132-145.

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cation neutron capture therapy are: first, there must be a large concentration of  $^{10}\text{B}$  in all areas of the neoplasm (15 mg  $^{10}\text{B}$ /kg of tumor<sup>3</sup>). This requires that the boron compound be injected intravenously and not directly into the suspected tumor area since the precise dimensions and configuration of the invasive neoplastic process is not clearly delineated. Second, a source must be available to irradiate the neoplastic area with a sufficient number of thermal neutrons to cause cellular destruction ( $10^{12}$  neutrons/cm<sup>2</sup>).<sup>4</sup> Third, a sufficiently large concen-

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(4) L. E. Farr and T. Konikowski, Inter. Jour. of App. Rad. and Isotopes, 19, 459 (1968).

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tration differential of  $^{10}\text{B}$  between the neoplasm and adjacent normal tissue must exist to permit the complete eradication of the tumor without adversely affecting tissue surrounding the neoplasm.

The primary emphasis for all of the previous studies of neutron-capture therapy is the syntheses of boron compounds which exhibit suitable structure-activity relationships for specific binding to neoplasms. This approach has met with only minimal success in fulfilling the requirement

for neoplasm destruction.<sup>5</sup> The possibility of using  $^{10}\text{B}$ -containing

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(5) A. H. Soloway, H. Hatanaka, and M. A. Davis, J. Med. Chem., 10, 714 (1967).

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antibodies for use in neutron-capture therapy has been suggested.<sup>2</sup> The basis for this approach is that antibodies are formed in response to the administration of an antigen and react specifically with that antigen. Consequently, if  $^{10}\text{B}$  could be incorporated into a tumor specific antibody protein, the antibody would subsequently concentrate the boron in the region of the tumor cell antigen and the cell might then be destroyed by subsequent thermal neutron radiation. This paper describes our initial exploratory research based upon this concept and provides encouragement for future work.

#### Results and Discussion

BSA-anti-BSA. The chemical modifications required for  $^{10}\text{B}$  incorporation are based on the extensive research which has been performed in the area of fluorescent protein tracing.<sup>6,7</sup> Proteins, including serum

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(6) "Fluorescent Antibody Techniques and their Applications," Akiyoshi Kawamura, Jr., Ed., University of Tokyo Press, 1969.

(7) R. L. Nairn, "Fluorescent Protein Tracing," 3rd Edition, The Williams and Wilkins Company, 1969.

antibodies, can be labeled with fluorescent dyes without material effect on their biological properties. The fluorescent proteins are then detectable in tracing experiments by fluorescence microscopy. Tracing may be carried out directly by injection of labeled proteins and subsequent histological examination, or by the application of immunological principles in which labeled antibody is used as a specific histochemical stain for antigenic materials in tissues.

Our first investigation of the applicability of this method used the standard precipitin reaction.<sup>8</sup> The purpose of this experiment was to

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(8) D. H. Campbell, J. S. McGarvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology," W. A. Benjamin, Inc., New York, 1964.

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determine if a boron-containing compound could be attached to an antibody protein under sufficiently mild conditions so as to have minimal effect on the resulting labeled antibody specificity. The modification of the antibody was based on results of anti-hapten antibody labeling.<sup>9</sup>

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(9) D. Pressman and A. L. Grossberg, "The Structural Basis of Antibody Specificity," W. A. Benjamin, Inc., New York, 1968.

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The boron compound which served as the precursor in subsequent diazo

coupling reactions was 1-(4-aminophenyl)-1,2-dicarba-closo-dodecaborane(12) which contained a natural abundance of boron isotopes (20%  $^{10}\text{B}$ ) and was converted to the corresponding diazonium ion for use as a labeling reagent containing a high total boron content. The preparation of this compound has previously been reported,<sup>10</sup> and the

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(10) M. F. Hawthorne, T. E. Berry, and P. A. Wegner, J. Amer. Chem. Soc., 87, 4746 (1965).

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synthesis is outlined in Figure 1. The diazonium salt of this compound

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Fig. 1

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could couple to antibody proteins through tyrosine, histidine and lysine groups.<sup>9</sup>

The 7S fraction of rabbit anti-bovine serum albumin (anti-BSA) was coupled with the diazonium salt in a phosphate buffered (pH = 8.0) aqueous solution at 4°. The resulting yellow ( $\lambda_{\text{max}} = 350 \text{ nm}$ ) boron-labeled antibody solution was purified and the quantitative precipitin reaction was performed on the various preparations. Two labeled antibody solutions were prepared by varying the molar ratio of the diazonium salt to the antibody. The first was prepared at a molar ratio of 20/1; the second with a ratio of 100/1. The quantitative precipitation curves are

shown in Figure 2. These results indicated that about 0.4% B (natural

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Fig. 2

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isotopic abundance) had been incorporated into the protein system with a concomitant loss of about 25% of the precipitating ability of the antibody.

These preliminary results with the BSA-anti-BSA system proved that suitable boron-containing compounds can be incorporated into antibody proteins under sufficiently mild conditions such as to maintain the specificity of the antibody.

Human Lymphocyte System. Since nearly all neoplastic cells thus far studied in detail appear to possess tumor associated antigens which induce an immune response in competent hosts,<sup>11</sup> cancer research

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(11) G. Klein, Federation Proceedings, 28, 1739 (1969).

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should benefit by the development and exploitation of this technique.

Neutron-capture therapy using  $^{10}\text{B}$ -conjugated antibodies allows a new approach to cancer therapy; one that combines the great tumor specificity of antibodies with the known  $^{10}\text{B}$  thermal neutron capture reaction. This method would thus limit and localize the destruction of tissue to tumor cells which carry  $^{10}\text{B}$ -labeled tumor specific antibodies at the time of

neutron irradiation.

Human histocompatibility antigens are also cellular antigens and invoke antibodies when introduced into an allogeneic host. Since more is known about the antigens and antibodies formed in multiparous females, the transplantation system was adopted as a model for the development of the test. The test for lymphocyte killing was adapted from Terasaki's lymphocytotoxicity test and possesses the advantage of microassays. It is presently used in most histocompatibility testing.<sup>12</sup>

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(12) P. I. Terasaki and J. D. McClelland, Nature, 206, 998 (1964)

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To ascertain the feasibility of in vitro lymphocyte killing by the alpha particle emitted from neutron irradiated  $^{10}\text{B}$ , purified peripheral blood lymphocytes were tested in boron-containing solutions of varying concentrations. In this test the boron was added as  $\text{K}_2^{10}\text{B}_{10}\text{H}_{10}$ , a compound which exhibits a low toxicity<sup>13</sup> ( $\text{LD}_{50} = 1025 \text{ mg/kg}$ ) and non-

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(13) E. L. Muetterties, J. H. Balthis, Y. T. Chia, W. H. Knoth, and H. C. Miller, Inorg. Chem., 3, 444 (1964).

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specific absorption to cellular material. Similar studies using sodium pentaborate in vivo have been reported.<sup>4</sup> The thermal neutron source was the UCLA R-1 100KW nuclear reactor with a core flux of  $1.5 \times 10^{12}$  neutrons/cm<sup>2</sup>-sec.



The results of the neutron radiation experiment using lymphocytes and  $K_2B_{10}H_{10}$  are shown in Figure 3.

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Fig. 3

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For the initial studies with boron-conjugated antibodies, a strong multispecific antiserum was selected. Among the specificities possessed by this antiserum were HL-A 1, 2, 3, 9, and Te55. Since nearly 50% of the population carry the HLA-2 antigen, lymphocytes needed for the tests were relatively easy to obtain.

The 7S fraction of the serum was isolated by passage of the serum through Sephadex G-200. The coupling reaction between the antibody containing fraction and the boron-containing diazonium compound was allowed to proceed at 4° in an aqueous phosphate buffered saline (pH = 7.4) media. The antibody was purified and analyzed for total boron (0.2%).

The antibody preparation was tested against cells which carried the HL-A specificity. The results of the neutron radiation experiments which varied both the antibody concentration and radiation time are shown in Figure 4. The initial antibody concentration was 15 mg/ml.

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Fig. 4

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These results indicate that the binding of the boron-conjugated specific antibody brought the boron atoms close enough to the cells so that the  $\alpha$ -particle disintegration product formed from neutron capture by  $^{10}\text{B}$  destroyed the lymphocytes. That the conjugation of the boron-containing label to antibody molecules did not affect the antibody specificity was shown by the cytotoxic effect in the test with complement employing this antibody preparation. The complement cytotoxic test using normal and boron-labeled antibodies gave similar results for the antibody activity.

The results obtained by varying the total boron concentration and the radiation time shown in Figures 3 and 4, were compared in order to demonstrate the ability of the antibody to concentrate the boron-containing label on the cell. From Figure 3,  $2\ \mu\text{g B}/\mu\text{l}$  gave 30% survival after 2 minutes of neutron radiation. From Figure 4, an antibody titer of 1:8 gave 30% survival after 2 minutes of radiation. The antibody contained 0.2% B. The concentration of the antibody and boron at this dilution (1:8) was  $1.87\ \mu\text{g Ab}/\mu\text{l}$  and  $3.8 \times 10^{-3}\ \mu\text{g B}/\mu\text{l}$ . Comparison of the total boron concentration required to give a comparable level of cellular destruction in the two experiments indicates that if the boron is first incorporated into a specific antibody the boron is approximately 500 times more effective in causing cellular destruction than a non-specifically bound boron compound.

Mouse Histocompatibility System. Antibodies directed against the C57 Bl10 mouse strain were prepared in the C3H mouse strain. The C3H anti-C57 Bl10 antibody (7S fraction) was conjugated with the boron-containing labeling reagent in the usual manner. The boron-labeled antibody solution containing 20% of available boron as  $^{10}\text{B}$  was then incubated with C3H lymphocytes and C57 Bl10 lymphocytes. The cells were washed with tissue culture media and subjected to neutron radiation. The results obtained are shown in Figure 5. The C3H lymphocytes survived the radiation since the boron-containing antibody was completely removed by washing with tissue culture media. The C57 Bl10 lymphocytes exhibited a marked decrease in survival owing to the specific binding of boron-containing antibody to the histocompatibility antigens against which the antibody was directed. This procedure of removal of excess antibody prior to radiation experiments indicates that specific antibodies, when labeled with  $^{10}\text{B}$ , will destroy only those cells which exhibit the antigen necessary for binding the boron-labeled antibody. Thus, in principle, the excess boron-containing antibodies can be removed prior to radiation of cancer patients in order to limit cellular destruction in non-neoplastic regions.

Cellular Incorporation of Elemental Boron. An additional mechanism which is operative in the immune response is the promotion of phagocytic

activity.<sup>14</sup> Phagocytosis is performed by polymorphonuclear leukocytes

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(14) W. S. Hammond in "Biology of the Immune Response," P. Abramoff and M. LaVia, Ed., McGraw-Hill, 1970, Chapter 13.

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and macrophage cells; the latter include monocytes in the bloodstream, the lining of cells of the sinusoids of the liver, bone marrow, lymph nodes, and spleen. It was anticipated that owing to increased phagocytic activity it would be possible to incorporate elemental boron within the cell owing to its particulate nature.

Elemental boron (natural isotopic abundance) (1-10 $\mu$  particle size) was added to Walker 256 cloned cells in tissue culture media. After 2.5 hours 78% of the cells exhibited visible boron incorporation within the cell. The cells containing the boron were removed with trypsin and replated, washing away the excess boron particles which had not been absorbed. One-half the cells were subjected to a 30 minute neutron irradiation, the other half were used as a control. Immediately following the neutron radiation both sets of cells appeared normal and no differences were noted under an inverted microscope with 40X objective. However, after 21 hours the cells subjected to neutron radiation had fragmented and were dead, whereas the boron-containing cells which had not been irradiated were normal. In a separate control experiment it was determined the W256 cells suffered no ill effects when subjected to 1 hour of neutron radiation.

The difference in the time limit for neutron radiation between peripheral blood lymphocytes and W256 cells reflects only a major difference in the radio-sensitivity of cells.<sup>15</sup> In addition, the alpha particle

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(15) A. P. Casarett, "Radiation Biology," Prentice-Hall, Englewood Cliffs, N. J., 1968.

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produced from <sup>11</sup>B fission causes observable damage to lymphocytes immediately, while the alpha particle damage in W256 cells is more delayed.

### Conclusions

These results obtained using boron-labeled antibodies and elemental boron in natural abundance strongly suggest that the concept of incorporating boron into proteins or polymers which exhibit marked tumor specificity is of great potential for use in neutron-capture cancer therapy. One additional and important aspect of the use of boron-labeled antibodies is that the antibodies when bound to the cell define the region of cellular destruction so that the exact dimensions of the neoplasms do not need to be determined as in other forms of radiation treatment.

The boron-containing labeling compound used in this study and the method of conjugation to the protein had little effect on the specificity of the antibody and exhibited no cellular toxicity at the concentrations used in these studies. In addition, use of highly <sup>10</sup>B enriched labeling

compound could increase the effectiveness of cellular destruction by as much as fivefold.

### Experimental

1-(4-aminophenyl)-1,2-carborane was prepared by the literature procedure.<sup>10</sup> Nitrogen and boron analyses were performed using standard analytical procedures.<sup>16,17</sup> Protein concentrations were determined

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(16) G. Ingram, "Organic Elemental Microanalysis," Reinhold, New York, 1962, Chapter 3.

(17) A. Kaczmarczyk, J. R. Messer, and C. E. Pierce, Anal. Chem. 43, 271 (1971).

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spectrophotometrically.<sup>18</sup> The neutron radiation experiments were per-

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(18) T. H. The and T. E. W. Feltkamp, Immunology, 18, 865 (1970).

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formed in the thermal column of the UCLA R-1 reactor. The thermal neutron flux with the reactor operating at 100 KW is  $1.5 \times 10^{10}$  neutrons/cm<sup>2</sup>-sec, which was determined by standard gold foil radiation techniques.

Diazotization of 1-(4-aminophenyl)-1,2-carborane. A general procedure was used for diazotization and suitable aliquots of aqueous solutions of

the diazonium salt were used in subsequent labeling experiments.

1-(4-aminophenyl)-1,2-carborane (24 mg, 0.1 mM) was added to 0.3 ml of 1 M aqueous HCl in an ice bath. A sodium nitrite solution (7 ml, 1 mg/ml) was added and the mixture allowed to stir for 1/2 hour. The solution was filtered and diluted to 25.0 ml with 0.01 M aqueous HCl. Owing to the reactive nature of the diazonium salt, the solutions must be kept cold and slightly acidic to retard decomposition. The diazonium solution was stable for at least 3 hours if kept in an ice bath.

The concentration of the diazonium salt was determined spectrophotometrically. A suitable aliquot ( $\sim 10 \mu\text{l}$ ) of the above solution was added to a 0.01 M (10 ml) solution of  $\beta$ -naphthol in 95% ethanol. After 30 minutes the absorbance was determined at 485 m $\mu$  ( $\epsilon_{485} = 1.98 \times 10^4$ ).

BSA-anti-BSA Reaction. Bovine serum albumin (BSA) and the 7S fraction of rabbit anti-bovine serum albumin (lyophilized) were obtained commercially and used as received.<sup>19</sup> All solutions were prepared in an

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(19) Nutritional Biochemicals Corporation, 26201 Miles Road, Cleveland, Ohio, 44128.

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aqueous phosphate buffer (pH = 8.0). The precipitin reaction was performed by the literature method.<sup>8</sup> The initial concentrations of BSA and anti-BSA were 10 mg/ml and 15 mg/ml, respectively.

Two separate boron-labeled anti-BSA solutions were prepared by varying the molar ratio of the diazonium salt to anti-BSA during the coupling reaction. The labeled anti-BSA solution obtained from the coupling reaction with a molar ratio (diazonium/anti-BSA) of 20/1 (II) and 100/1 (III) were used in the precipitin reaction. A third precipitin reaction was performed using BSA and unmodified anti-BSA (I).

Suitable aliquots of the diazonium solution were added to the anti-BSA solution at 4<sup>0</sup> at pH 8.0. The pH was maintained at 8.0 by dropwise addition of 0.1 M NaOH. The resulting yellow solution ( $\lambda_{\text{max}} = 350 \text{ m}\mu$ ) was allowed to stir for 24 hours at 4<sup>0</sup>. The mixture was centrifuged to remove the decomposed diazonium salt which had not coupled to the anti-BSA. The clear yellow supernatant was dialyzed for 48 hours against phosphate-buffered saline. The solution was evaporated to the original volume and the precipitin reaction performed.

Human and Mouse Histocompatibility Boron-labeled Antibodies. The 7S fraction of serum was obtained by gel filtration through Sephadex G-200 with phosphate-buffered saline. The 7S fraction was concentrated by negative pressure dialysis.

The labeling experiments were performed as outlined previously using a molar ratio of 10 diazonium/1 antibody. The coupling reaction was allowed to proceed for 3 hours at 4<sup>0</sup> in a phosphate-buffered saline solution (pH = 7.4). The yellow solution was passed through Sephadex G-25 with phosphate-buffered saline to remove the unreacted diazonium salt. The effluent was again concentrated by negative pressure dialysis.



Boron-labeled antibody solutions (1  $\mu$ l) of different concentrations were added to the wells of a microtest plate<sup>20</sup> under 5  $\mu$ l of mineral oil.

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(20) Falcon Plastics, Los Angeles, California.

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The oil is added to the well first to prevent the minute amounts of reagents from drying. A thousand purified, peripheral blood lymphocytes suspended in 1  $\mu$ l of McCoy's medium were added to each well. Following neutron radiation, eosin red was added to each well and the test terminated by fixation of the cells with formalin. The tray was prepared for reading in an inverted phase microscope. Live cells in this test appear bright, clear and unstained while dead cells are dark and diffuse.

Lymphocytotoxic assays were performed on all boron-labeled antibody solutions. After one-half hour incubation of the cells with the antibody, 5  $\mu$ l of rabbit complement was added to each well followed by an additional one hour of incubation. Eosin red was then added to each well to stain dead cells and the test was fixed with formalin. Each of the boron-labeled antibody preparations gave an antibody activity titer identical to the unmodified antibody.

For neutron radiation experiments with the excess boron-labeled antibody removed (mouse histocompatibility system), boron-labeled antibody solutions at full strength were allowed to react with cells for one hour. The cells were then centrifuged and washed twice in tissue culture media. Antibody-coated lymphocytes were then adjusted to give

1000 cells in 1  $\mu$ l and dispensed into the wells of a microtest plate under oil. The plates were treated in the same manner as described previously.

Intercellular Incorporation of Particulate Boron. Six control and six experimental small flasks were each plated with  $0.28 \times 10^6$  Walker 256 cloned cells. The cells in the experimental flasks were allowed to take up boron particles from a saline solution added to their medium. The cells were left in contact with the particulate boron for 2.5 hours and then removed with trypsin and replated on small flasks. Seventy eight percent of the cells had visible uptake boron. About 40 minutes after the small flasks were incubated at  $37^{\circ}$  in growth medium containing 20% fetal calf serum, the cells were subjected to neutron radiation for 30 minutes. The controls which had no boron incorporation showed no change after the radiation. Three flasks containing cells with boron were not irradiated to determine any toxic effects of the boron, the three other flasks with boron were irradiated for 30 minutes. All flasks were examined following the radiation experiment and no differences were noted under inverted scope with 40X objective. An additional 2 ml of regular growth medium was added to each flask before replacing them in the incubator. After 21 hours both sets of control cells (no boron and irradiated, and boron with no irradiation) all appeared normal, while the cells with boron and irradiation were all now fragmented, floating and dead.

Acknowledgements

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Preparation of Boron-Labeling Reagent

Figure 1

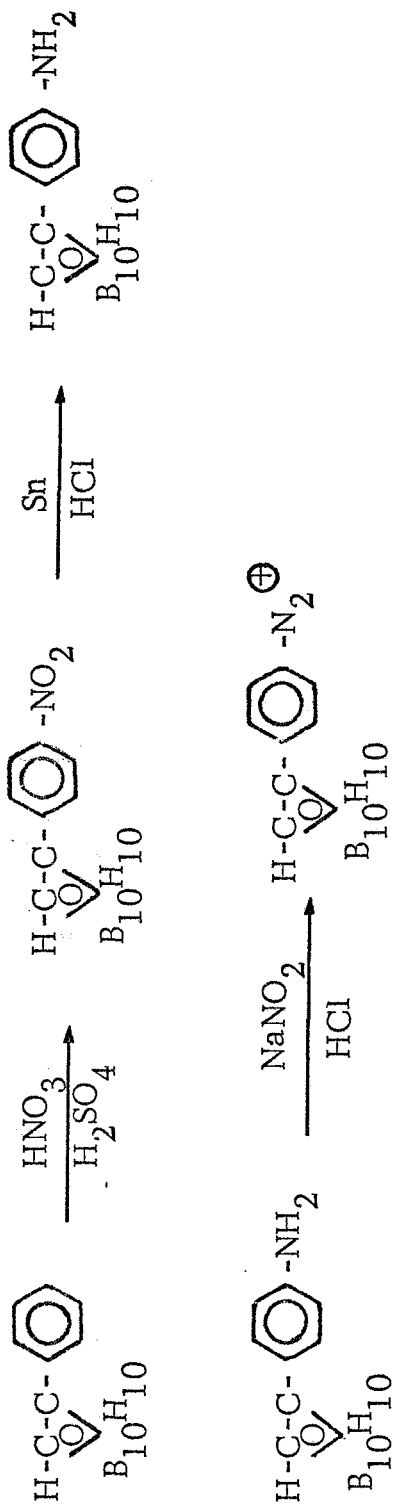


Figure 2. Precipitin reaction between bovine serum albumin (BSA) and rabbit anti-bovine serum albumin (anti-BSA).

- I BSA + 7S anti-BSA
- II BSA + 7S anti-BSA (labeled) complex containing 0.35% B
- III BSA + 7S anti-BSA (labeled) complex containing 0.38% B

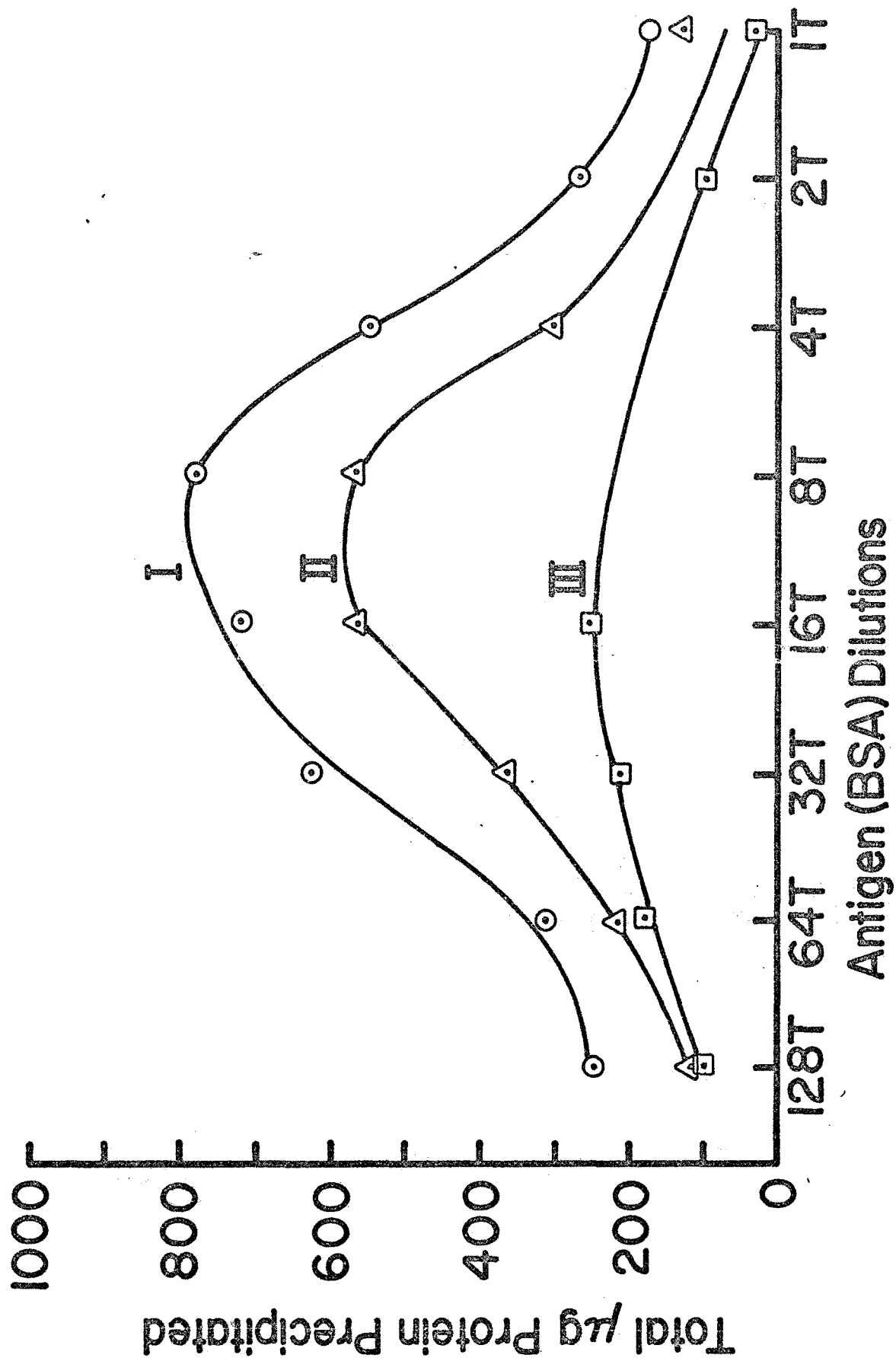


Figure 3. Survival curves for peripheral blood lymphocytes with  
 $K_2B_{10}H_{10}$  solutions.

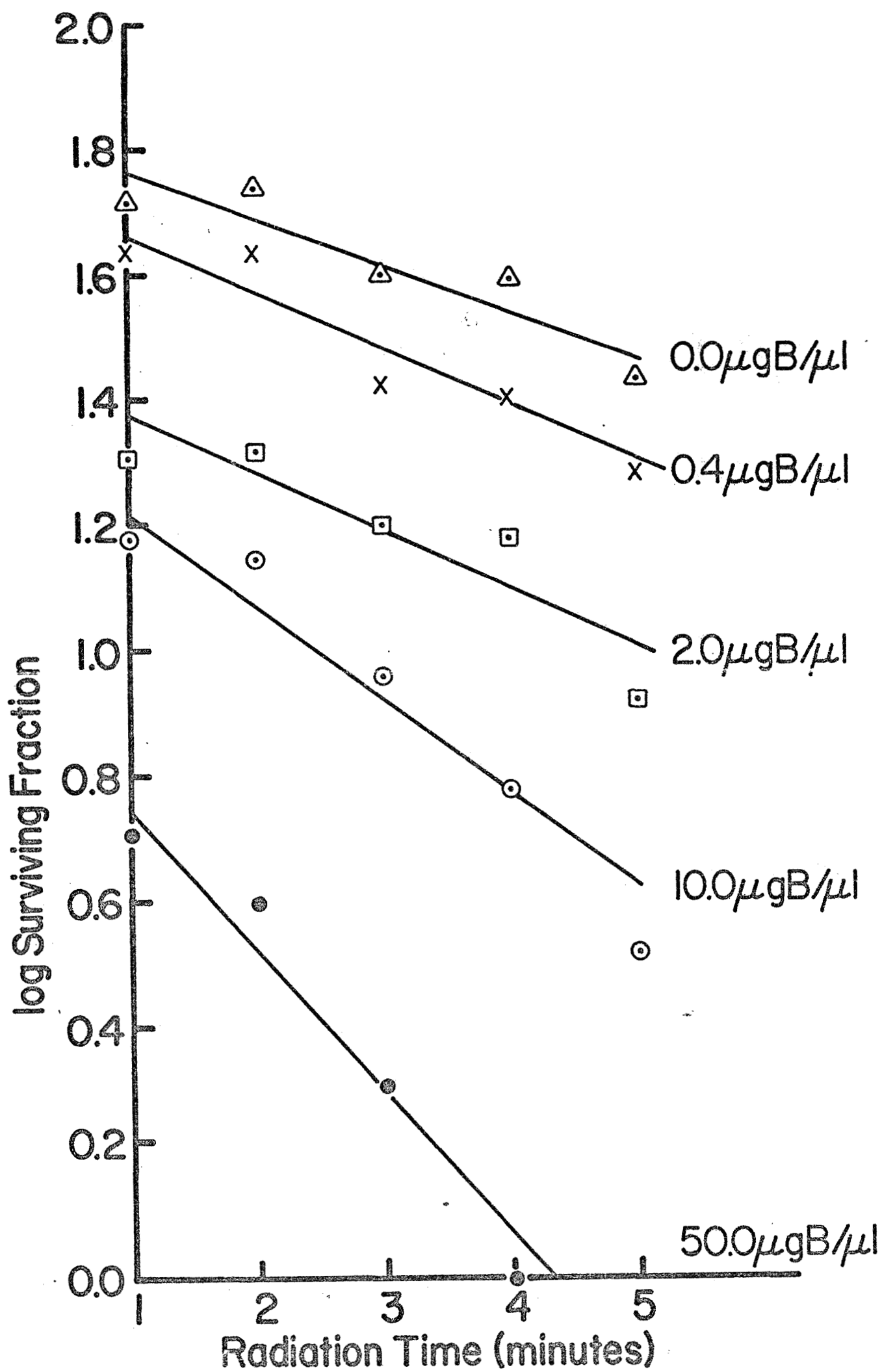




Figure 4. Effect of titration of boron-labeled antibody on survival of peripheral blood lymphocytes. Initial antibody concentration = 15 mg/ml; 0.20% B.

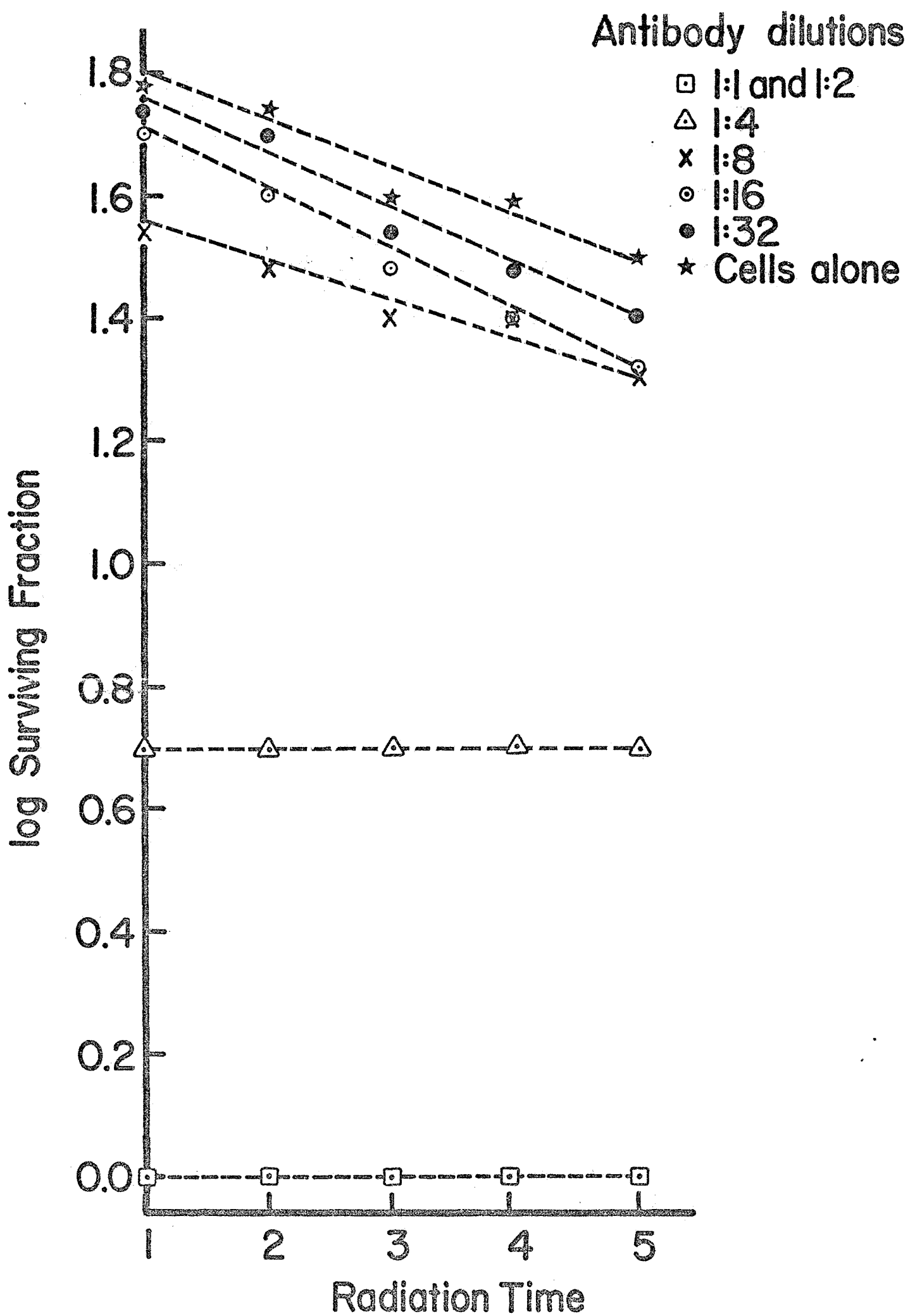


Figure 5. Survival of C3H and C57Bl10 peripheral blood lymphocytes after incubation with boron-labeled C3H anti-C57Bl10 antibody.

