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EXPOSURE OF BIOLOGICAL SPECIMENS TO HIGH FLUXES OF THERMAL NEUTRONS

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ABSTRACT

The background for proposed biological experiments using thermal neutrons is reviewed. The number of thermal neutrons/cm² necessary to obtain an average of one transmutation of N¹⁴ per bacteriophage is estimated. It appears for N¹⁴ capture that approximately 2.25×10^{17} thermal neutrons/cm² will be required to induce an average of one transmutating event in phage deoxyribonucleic acid.

Besides the neutrons effecting N¹⁴(n,p)C¹⁴, other radiations from a reactor are listed that might induce phage inactivation; it appears that the most damaging of various fission or capture radiations are capture-gamma radiation from the phage environment and the 0.6-mev proton from the n,p reaction of N¹⁴.

Preliminary experiments are described that were performed in facilities in the ORR, the LITR, and the MITR. At present, the known limiting factor for reaching the level of an average of one transmutation of N¹⁴ per bacteriophage without excessive inactivation from other sources is the high background capture-gamma radiation although other radiations may present problems too. The capture-gamma radiation can be decreased by a factor of 80 times by construction of a special facility (ORNL-TM-399) and the effect of the capture-gamma and other radiations can be decreased by the use of chemical protective agents. It is expected that together these changes in exposure of biological specimens may make the thermal neutron project a practical adjunct to other techniques in molecular biology.

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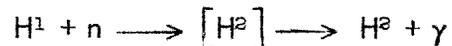
EXPOSURE OF BIOLOGICAL SPECIMENS TO HIGH FLUXES OF THERMAL NEUTRONS

INTRODUCTION

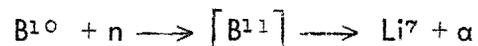
This presentation is a discussion of the possible use of different neutron cross-sections of isotopes as an analytical tool in biology. The isotopes of nitrogen, N^{14} and N^{15} , are of particular interest since N^{14} has a cross-section to thermal neutrons approximately 10^5 times that of N^{15} . Organisms or specified molecules of organisms now can be made of these two isotopes of nitrogen, and it is believed that, under proper conditions, biological effects from transmutation events per se may be recognizable.

The cytogenetic effect of thermal neutrons was demonstrated for the first time on chromosomal aberrations in Tradescantia¹, and it was shown that hydrogen, nitrogen, and boron are responsible for about 99% of the dose produced by neutron capture. A total of 83% of this dose can be attributed to protons and alpha rays from nitrogen and boron-capture reactions. Hydrogen has a significant effect because it is present in high molar concentration in tissue, and boron is effective because it has a very large cross-section for thermal neutrons. The chromosomal aberrations were not the direct result of the transmutation events but were caused by ionizations from the emitted alpha particles, gamma radiation, and protons from the n,α , n,γ , and n,p reactions.

The capture reactions of these elements for thermal neutrons are as follows:

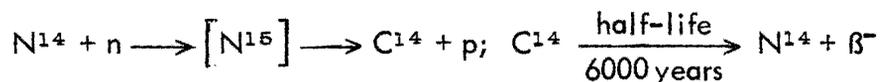


The cross-section for H^1 is 0.33 barns (1 barn = 10^{-24} cm²)



The cross-section for B^{10} is about 4017 barns, but the effective cross-section of the natural mixture of B^{10} and B^{11} isotopes is about 715 barns.

The cross-section for N^{14} is $1.75 \text{ barns} \pm 0.05$.



Thermal neutrons were used by Conger² on Tradescantia that had been enriched by growing them in B^{10} solutions. The chromatid aberration frequency was five times that of the nonenriched controls following thermal neutron treatment.

N^{15} has a cross-section to thermal neutrons of $24 \pm 8 \mu\text{barns}$. N^{15} enrichment to decrease the sensitivity of a living organism to thermal neutrons was first done for survival of Neurospora conidia³. At that time N^{15} was available only at a purity of 65%, but this was sufficient to demonstrate a dose-reduction in killing effect of about 0.5 in the N^{15} -enriched conidia. At the present time N^{15} is readily available at 97.5% purity, and 99.99% purity can be obtained at reasonable cost.

Stent and Fuerst⁴ demonstrated that P^{32} -labeled phages during a certain part of the phage life cycle are immune to killing by transmutation even when growing in P^{32} -labeled bacteria. A series of experiments by Stent and his collaborators have firmly established that before DNA synthesis begins — but coinciding with synthesis of a protein — P^{32} -labeled DNA passes through a stage not sensitive to transmutation of P^{32} (ref. 5).

It occurred to Atwood (personal communication) that the Stent effect could be studied by transmutation of N^{14} molecules in phage made principally of N^{15} . DNA, RNA, protein, and their parts could be labeled individually and together and followed throughout the life cycle. At the time Atwood first suggested this experiment (1955), it was clear to him that the only reactor in which the experiment might be feasible was the MTR in Idaho; also high-purity N^{15} was unavailable at that time. No experiments were performed.

In late 1959 and early 1960, in Pavia, Italy, Calef and von Borstel discussed the possible uses of the thermal neutron method. These suggested experiments present many technical problems in themselves; they are not meant to imply that answers would be quick and easy, only that the thermal neutron method could turn out to be a useful adjunct technique for many different basic biological problems. One of the first ideas

was that the method could possibly be used to find the sequence of nucleotides in a gene, an extension of a suggestion by Krieg (personal communication) that N^{14} purines and pyrimidines be replaced by bases made from N^{15} .

1. Nucleotide sequence. Assuming that one nucleotide exists for each site in the rII cistron of phage T4, if the various bases made of N^{14} were put in the N^{15} genome and exposed to thermal neutrons, then when mutants were analyzed, "hot spots" for mutations would appear at different sites, dependent upon the N^{14} base used. If more than one nucleotide were present at a site, then distribution of nucleotides could be found.

2. Information transfer. N^{14} deoxyribonucleic acid (DNA) could be used to make N^{15} DNA. The N^{14} DNA could be destroyed, and then the N^{15} strands could be used to make N^{14} or N^{15} DNA again. Thus, under appropriate conditions, mixtures of newly synthesized N^{15} DNA and the old N^{14} DNA could be separated by the inactivation of the N^{14} DNA.

3. Messenger ribonucleic acid (RNA). The Volkin and Astrachan^{6,7} experiments could be repeated so that all nitrogen-containing molecules would be N^{15} except the messenger RNA. This could be systematically studied with thermal neutrons, one base at a time.

4. Influence of different molecules on cell viability. The entire architecture of phage could be studied at different phases of the life cycle, amino acid by amino acid, base by base. Atwood was the first to suggest this straightforward, long-term experiment.

5. Analysis of active centers. The active centers of any enzyme from any organism capable, practicably, of growing on N^{15} could be studied by putting in different N^{14} amino acids and assaying the enzyme activity after exposure to thermal neutrons. An additional advantage is that it would be unnecessary to have pure enzymes for active-center analysis.

6. Combinational analysis. β -galactosidase, phosphatase, tryptophan synthetase, and lysozyme are possible enzyme systems wherein the genes, the messengers, and the enzymes themselves could all be studied by thermal neutrons.

ESTIMATED TRANSMUTABILITY

The first question is whether thermal neutrons could be made available in sufficient quantity so that a biologically meaningful effect could be recognized in viruses. A minimum of one transmutational event of N^{14} for the DNA of the bacteriophage, T4, will be used to estimate a reasonable number of thermal neutrons cm^{-2} required.

The rate at which nitrogen nuclei of a phage are transmuted is

$$\text{hits/sec} = N\sigma\phi \quad (1)$$

where N is the number of nitrogen atoms in a phage, σ is the cross-section of N^{14} in cm^2 , and ϕ is the thermal neutron flux ($\text{cm}^{-2} \text{sec}^{-1}$).

Therefore, the number of thermal neutrons per cm^2 necessary to reach an average of one transmutational event per phage DNA can be stated as

$$n/\text{cm}^2 = \frac{1}{N_{\text{DNA}}\sigma} \quad (2)$$

where n is the number of thermal neutrons and N_{DNA} is the number of nitrogen atoms in the DNA of phage.

The base pair adenine-thymine contains seven nitrogen atoms while the base pair guanine-cytosine contains eight. There are two phosphorus atoms per base pair. Hence, the number of nitrogen atoms in the DNA of a phage particle can be calculated from

$$N_{\text{DNA}} = \frac{P_{\text{DNA}}(7 + f)}{2} \quad (3)$$

where P_{DNA} is the number of phosphorus atoms per phage and f is the fraction (on a molar basis) of guanine-cytosine base pairs, and thus the fraction of the difference in number of nitrogens per base pair between adenine-thymine and guanine-cytosine.

Both f and P_{DNA} have been accurately determined:

$$P_{\text{DNA}} = 3.62 \times 10^5$$

(back calculated from Rubinstein et al.⁸), and

$$f = 0.34$$

(Wyatt and Cohen⁹). Therefore $N_{\text{DNA}} = 1.33 \times 10^6$, a number which, interestingly enough, is exactly 1% of the molecular weight of the bacteriophage DNA.

Although the cross-section to thermal neutrons (σ) for N^{14} is 1.75 barns, when the absorption cross-section at all energies for neutrons from the Oak Ridge Graphite Reactor was determined (Binford, personal communication), the effective cross-section of N^{14} was 3.3 barns (σ_{eff}).

Substituting in equation (2), then, we find that the number of thermal neutrons/cm² to effect a transmutational event in phage DNA is

$$n/\text{cm}^2 = \frac{1}{1.33 \times 10^6 \times 3.3 \times 10^{-24} \text{ cm}^2} = 2.25 \times 10^{17} \quad (4)$$

This number, $2.25 \times 10^{17} n/\text{cm}^2$, will be used as a guide for determining what constitutes an adequate source of thermal neutrons and for determining the approximate lengths of time that the phages must be exposed. In order to complete an experiment in a reasonable amount of time, a thermal neutron flux of not less than 5×10^{11} thermal neutrons $\text{cm}^{-2} \text{sec}^{-1}$ will be needed. For correct back-computations on amounts of DNA from an observed thermal neutron effect, accurate σ_{eff} determinations would have to be performed in the exposure facility.

INACTIVATION BY EXTRANEIOUS RADIATIONS

Besides the transmutation process of N^{14} per se, organisms exposed to thermal neutrons in a nuclear reactor will be subject to a variety of radiations, some extrinsic, and others intrinsic (i.e., coming from within the organism). The extrinsic radiations comprise (1) gamma-radiation from fission of U^{235} , (2) products from n,γ , n,α , and n,p reactions with nuclei in the environment, (3) protons from collision of fast and epithermal neutrons with hydrogen and (4) beta and gamma decay radiations from different isotopes formed during the exposure. The intrinsic radiations include (1) capture-gamma radiation, (2) protons from n,p and n,α reactions, (3) hydrogen-recoil protons, and (4) decay radiations from activated atoms.

1. Extrinsic radiations. The gamma radiation from fission can be effectively eliminated by shielding the samples with a dense material, such as lead or bismuth.

The capture-gamma radiation produced by materials in the environment of the phage is a factor of concern. This radiation is produced by the materials of which phage are made, by the suspension medium, and by the container. This factor can be reduced by suspending phage in media containing D_2O where viability is normal. The sample holders and the shielding material produce large amounts of capture-gamma radiation. The amount is difficult to compute accurately. Here selection of materials of low neutron and high photon cross-section can best reduce radiation effects¹⁰.

The fast and epithermal neutrons from the reactor will collide with hydrogen, which, in turn, will induce primary ionizations. To reduce fast neutron killing, samples should be kept as thin as possible, and specimens should be selected that can be kept dry. Also, materials selected for shielding against gamma radiation should be chosen for their efficiency in thermalizing neutrons.

In general, the decay radiation from newly formed isotopes in the environment can probably be neglected, since it will induce the least inactivation of any of the extraneous radiations. However, the sample holder itself should be constructed from properly selected material to minimize any possible effects.

2. Intrinsic radiations. Our estimates of the intrinsic radiation levels are based on little information and might be in error by a factor of 10.

Presumably most of the intrinsic radiation would be caused by the proton emitted by N^{14} following the capture of a neutron by N^{14} . This radiation could mask some or most transmutational effects, since the sought transmutational effect comes from the same reaction. The energy of the proton from the n,p reaction of N^{14} is 0.6 mev, and such a proton has a range of about 10^{-2} mm in water. A proton of this energy produces approximately 10^8 ion pairs/ 10^{-3} mm; about half of the ionizations can be considered to be from delta radiation. It can be estimated roughly that the effective radius of DNA in a T4 bacteriophage is about 50μ ¹¹. Therefore, approximately 50 ion pairs will be generated inside a phage from a 0.6-mev proton. Chemical protective agents that apparently enter the phage can be used to mitigate the effect of the ionizations without altering the transmutation frequency.

The mean lethal dose (dose for an average of one lethal event per phage, the $1/e$ dose) for T4 bacteriophage is about 50 krad¹². If gamma radiation induces approximately 2 ion pairs/ μ^2 /rad, then about 10 primary ionizations occur inside a phage before a lethal event occurs.

Thus for each transmutational event approximately five lethal events may be induced by the emission proton from the n,p reaction of N^{14} . This would reduce the signal-to-noise ratio of the sought transmutational events by a factor of 150. The recoil C^{14} nuclide from the n,p reaction of N^{14} will have an energy of 40 kev, enough to break covalent bonds, but not enough to induce any primary ionizations. Outside the phage of their origin, the different effects of lethality induced by emission protons and lethality induced by the transmutation per se can probably be accurately assayed by phage dilution experiments and use of different mixtures of N^{14} and N^{15} phages. Inside the phage of origin, the different effects can be attacked by use of the different isotopes within the phage, and by back-calculations of the expected and found amounts of DNA under different conditions.

The second most efficient inactivating radiation would appear to be that from the n,γ reaction of hydrogen. This is principally because hydrogen is the most common element in organic compounds, constituting roughly two-thirds of all atoms composing any organism. Conger and Giles¹ found that hydrogen capture accounted for about one-half the effect of that of N^{14} capture in Tradescantia chromosomes. This factor would be less in the DNA of an organism because of a lower hydrogen/nitrogen ratio than in tissue as a whole, and could also be decreased by making the organisms, at least partially, out of D_2O . The H^2 nuclide from neutron capture by H^1 will not recoil with enough energy to induce any primary ionization.

Conger^{1,2}, using chromosome breakage in Tradescantia as criterion, and Atwood and Mukai³, using conidial spore inactivation in Neurospora, found that boron (naturally occurring in Tradescantia and added in the case of Neurospora) contributed considerably to effects produced when organisms were exposed to a thermal neutron flux. No boron has been found in phage by careful spectroscopic analysis, but undetected traces, if present, could possibly enhance the background radiation.

PRELIMINARY EXPERIMENTAL DEVICES

Upon return of von Borstel from Italy in the autumn of 1960, contact was made with the Reactor Operations Division. Colomb joined the project since considerable experience with reactor physics was requisite for even the preliminary experiments.

The first exposures were made in the Oak Ridge Research Reactor (ORR) during mid 1961. A plug was made for the Low Intensity Test Reactor (LITR) and was put into operation in December 1961. Calef came to Oak Ridge in late 1961 to help develop the biological methodology and worked here on the project until March 1962, when he returned to Italy.

1. Oak Ridge Research Reactor. The first exposures of bacteriophage to thermal neutrons were made in a closed Sugarman container (a large, cylindrical, thick-walled, lead vessel) lowered to within a few inches of the reactor elements for 12 hours. The phage were suspended in a liquid medium, compressed flat in a holder made of zirconium foil.

The experiments were exploratory in nature, designed to see if viable phage could be recovered after exposure to a high flux of fast neutrons, a reasonably high thermal neutron flux, with some protection from fission gamma radiation in the Sugarman container. Viable phage were found in one experiment only, where apparently the gamma-heating of the lead container was kept from getting too high because the temperature of the reactor pool was low. In two subsequent exposures no viable phage were recovered. This experiment was unwieldy and virtually impossible to accurately monitor for dose. It was therefore discontinued, but in these experiments the total number of fast neutrons traversing the phage sample was at least a factor of 10 more than with any other experiment, and viable phage were recovered once.

2. Low Intensity Test Reactor. A plug for holding biological specimens was designed, built, and installed in the HB-2 hole of the LITR. This plug was simply a steel container with 1 foot of pure lead at the reactor end, with adequate cooling by water to keep the lead from melting, and adequate built-in shielding to protect the investigators. A long sample holder made of aluminum was inserted into the plug. The sample, in a small graphite box (usable volume, about 0.6 cm^3), was kept frozen by expansion of CO_2 under high pressure in the vicinity of the graphite box. All irradiations were made with the frozen specimens.

The maximum thermal neutron flux possible at the specimen position is 1.4×10^{12} thermal neutrons $\text{cm}^{-2} \text{sec}^{-1}$. This flux was decreased in certain experiments simply by irradiating the specimens at different measured distances away from the lead shield.

3. Massachusetts Institute of Technology Reactor. A few exposures were made in the MITR, a deuterium oxide reactor. The details of the irradiation procedure are given in the next section. A sample holder was constructed from pyrolytic graphite for these irradiations since the samples were exposed in the reactor a little above room temperature and a normal graphite container would have absorbed the phage suspension.

PRELIMINARY RESULTS

Considerable progress has been made in obtaining survival at high fluxes of thermal neutrons, but capture-gamma radiation has been found to be the limiting factor in achieving survival at higher fluxes.

While the number of thermal neutrons/ cm^2 can be accurately determined with relatively simple devices, it was found that by the commonly available methods accurate dosimetry of the gamma radiation was not possible. The calculable minimum amount of fission gamma in our experimental setup in the MITR was about 2000 rad/hour. An attempt was made to estimate the capture-gamma dose rate at the sample location. This computation was made assuming that the lead and the aluminum constituting the irradiation facility did not contain any impurities and, therefore, the calculated dose rate could be underestimated. The results of this computation indicate that a minimum dose rate of approximately 10^5 rad/hour has to be expected at the site of irradiation.

The amount of total gamma measured by an ionization chamber with a graphite wall approximately 2 inches thick was roughly 10^6 r/hour. By use of a standard suspension of phage exposed to a Co^{60} gamma source, we have made reference death-rate curves which, by comparison with those obtained in the reactor, gave an estimated maximum amount of gamma radiation of 7×10^6 r/hour in the experimental setup (Fig. 1). Within the reactor, or Co^{60} gamma source, no difference in rate of mortality was observable when either N^{14} or N^{15} phages were exposed. This indicates the observed killing was not from intrinsic capture reactions.

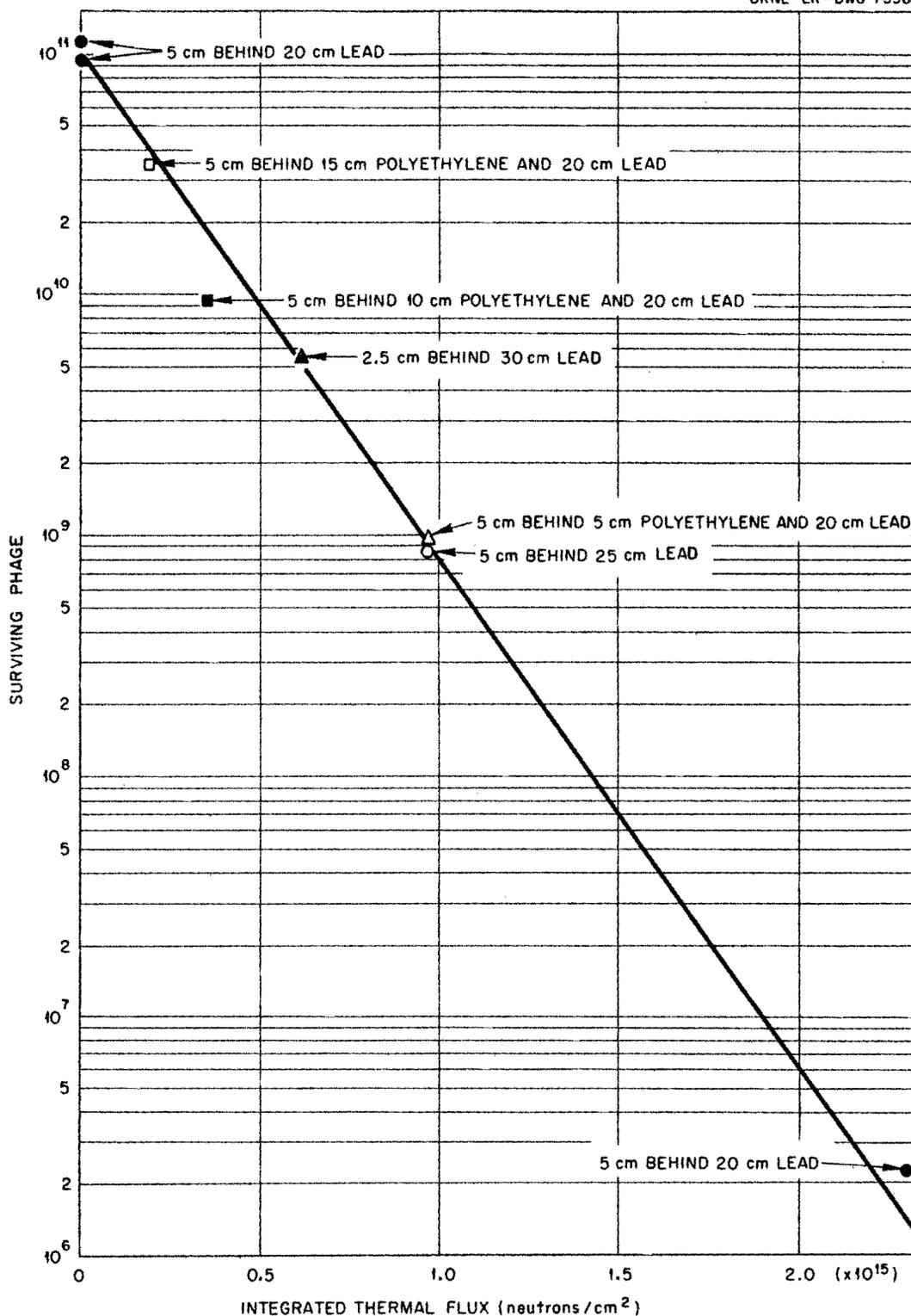
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Figure 2. Dose-action curve for exposure of frozen bacteriophage T₄ in the LITR with different amounts of added shielding.

The amount of killing of bacteriophage T4 in an exposure chamber near the face of the LITR is about 350 times higher than expected from calculated gamma radiation from fission of U^{235} in the reactor core. It is possible to conclude that most of the phage inactivation can be attributed to gamma radiation from thermal neutron capture in the phage environment. This was indicated by the fact that the killing was correlated directly with the thermal neutron flux independent of the amount of lead or polyethylene shielding added to the initial lead shielding (Fig. 2). Added lead was used to shield phage from fission-gamma radiation and added polyethylene was used as a moderator for fast neutrons and as a control for the added lead part of the experiment. In neither case did the points depart from the exponential curve toward the direction of increased survival, a result that would be expected if fission-gamma shielding had been ineffective or if the fast neutron flux had contributed to the inactivation of the phage.

Thus, the feasibility of the thermal neutron experiment appears to be conditioned by the ratio of thermal neutrons per cm^2 to gamma radiation in rad (nvt/ γ t). This ratio in the HB-2 hole of the LITR is equal to $(2.31 \times 10^{15} \text{ thermal neutrons per } cm^2) / (7.0 \times 10^6 \text{ rad}) = 3.3 \times 10^9$. This ratio simplifies comparison between different conditions of radiation shielding and between different reactors. It should be kept in mind that the value of this ratio for an efficient experiment involving phage should be in the order of 10^{12} . This is based upon the presumed possibility of detecting transmutation events under conditions of 1% survival of phage from background gamma radiation.

Having shown that most of the inactivation is due to capture-gamma radiation, we were interested to determine the nvt/ γ t ratio under conditions where capture gamma is minimized. A simple experiment was performed at the MITR, a deuterium oxide-moderated reactor¹³. Three bismuth plugs, 1 inch wide by 3 inches long, were placed between the phage and the reactor core; these were expected to stop only a fraction of the fission-gamma radiation because of geometrical configuration. Placement of efficient shielding was considered impractical and too expensive to do without a preliminary test. The nvt/ γ t ratio of this preliminary experiment was 7.6×10^9 , about twice as high as the HB-2 hole of the LITR. Most of the gamma radiation seemed to be fission-gamma, as indicated by the known flux within the reactor and by the rapid rate of phage inactivation. The gamma equivalent dose was in the range of 1.5×10^6 rad/hour.

PROSPECTS

To perform the envisioned biological experiments, if our assumptions are correct, it will be necessary to increase the $nvt/\gamma t$ ratio 50 to 100 times. Appropriate shields for fission-gamma radiation producing less capture-gamma radiation must be constructed. Such a shield has been designed. Computations indicate that this shield can improve the $nvt/\gamma t$ ratio approximately 80 times over that of the biology facility at the LITR.

In addition to the increase in $nvt/\gamma t$ ratio coming from shielding modification, chemical protection can also help to change the biological effectiveness of the ratio. Preliminary experiments with frozen phage (von Borstel and Bond, unpublished results) indicate that at least a 3-fold protection is possible with standard protective methods involving the sulfhydryl compound, cysteamine (mercaptoethanolamine).

Thus by both chemically protecting the biological material and by using the newly designed shielding device, it may be possible to increase the effective $nvt/\gamma t$ ratio a minimum of 250 times, a factor of at least 2.5 over the effective $nvt/\gamma t$ ratio believed to be necessary to achieve, and possibly measure biologically, an average of one transmutational event per bacteriophage from transmutation of N^{14} by thermal neutrons¹⁴.

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