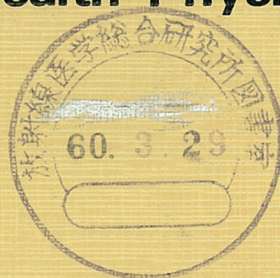
NIRS-M-52

Proceedings of the Second Workshop on Tritium Radiobiology and Health Physics



March 1985

Edited by

H. Matsudaira, T. Yamaguchi, and H, Etoh

—National Institute of Radiological Sciences—

Chiba 260 , Japan

NIRS-M-52

Proceedings of the Second Workshop on Tritium Radiobiology and Health Physics

March 1985

Edited by

H. Matsudaira, T. Yamaguchi, and H, Etoh

—National Institute of Radiological Sciences—

Chiba 260 , Japan

Proceedings of the Second Workshop on Tritium Radiobiology and Health Physics

October 30 - November 1, 1984

Published by
National Institute of
Radiological Sciences
9-1, 4-chome, Anagawa, Chiba-shi 260,
JAPAN

FOREWORD

The Second Workshop on Tritium Radiobiology and Health Physics (Exchanges SA-53 in the US-Japan Fusion Cooperation Program) was held from October 30 to November 1 , 1984, at the National Institute of Radiological Sciences,NIRS, in Chiba under the sponsorship of Science and Technology Agency in Tokyo and Department of Energy in Washington.

It is the pleasure of the executive committee to issue the proceedings which contains full papers presented at the meeting here in time. We would like to express our sincere thanks to all the participants for their active contribution to the workshop and to staffs of STA and NIRS for their collaborations in carrying out the meeting successfully. Special thanks are due to Dr. Gerald Goldstein in US DOE for his assistance in planning the Workshop.

We missed Dr. Roger A.Pedersen who unfortunately could not attend the meeting. It was with a deep sorrow that we were informed of the sudden death of Dr. S.L.Commerford , Brookhaven National Laboratory, who had been carrying out pioneering works in tritium metabolism.

We must acknowledge all the participants from US who have kindly assisted in editing many papers of Japanese participants. Dr. James D. Regan from Oak Ridge National Laboratory, who had been staying at NIRS on a grant from STA , also helped us in preparing the manuscripts. Several friends from European Communities and Canada expressed their interest in the meeting.

Finally we would like to thank Mr. M.Wada,STA, Dr.T.

Kumatori,Director-General and Dr. T.Terasima,deputy Director -General, NIRS, and Dr. G.D.Duda,US DOE, for their remarks at the meeting.

March 1, 1985

Hiromichi MATSUDAIRA,editor in chief

Takeo YAMAGUCHI

Hisami ETOH and

All Members of Division of Biology

Program

Date Time	Subject	Chairmen
Oct.30(Tue)		
9:30	OPENING Mr.M.Wada (STA)	Dr.H.Matsudaira (NIRS)
9:40	WELCOME Dr.T.Kumatori (NIRS)	
*SESSION I.	TRITIUM METABOLISM	
9:50	"Uptake and metabolism of tritium gas and vapor in the rat" Dr.Y.Ichimasa (Ibaraki Univ)	Dr.Y.Akita (Ibaraki Univ)
10:20	"Tritium metabolism in newborn mice" Dr.M.Saito (Res Reactor Inst, Kyoto Univ)	
10:50	BREAK	
11:00	"Studies on the tritium uptake in some edible plants and transfer to the rat" Dr.K.Arai (NIRS)	Dr.Y.Ujeno (Kyoto Univ)
11:30	"Metabolism of organically bound tritium" Dr.C.C.Travis (ONL)	
12:00	LUNCH	
*SESSION II.	MOLECULAR AND CELLULAR EFFECTS OF TRITIUM	
13:00	"Microdosimetry of tritium beta particles" Dr.A.Ito (Univ Tokyo)	Dr.O.Yamamoto (Nucl Res Inst, Hiroshima Univ)
13:30	"Tritium effects on DNA: Tritium concentration dependency of RBE in aqueous solution" Dr.T.Kada (Natl Inst Genet)	
14:00	"Radiobiological effects of tritiated water on yeast" Dr.T.Ito (Univ Tokyo)	
14:30	"Analysis at the molecular level of mutations induced by tritium, X-rays and chemical mutagens" Dr.W.R.Lee (Louisiana State Univ)	
15:00	BREAK	
15:15	"The RBE of tritium beta-rays for mutagenesis in mouse lymphoma cells under chronic irradiation conditions" Dr.N.Nakamura (RERF and Univ Tokyo)	Dr.S.Okada (Univ Tokyo)
15:35	"Mutation induction by tritiated water and effects of some agents in mouse leukemia cells" Dr.I.Furuno-Fukushi (NIRS)	
15:55	"Deuterium oxide inhibition of gamma ray dose-rate effects on growing mouse leukemia cells" Dr.A.M.Ueno (NIRS)	
16:25	"Malignant cell transformation induced by tritiated water in golden hamster embryo cells" Dr.O.Nikaido (Kanazawa Univ)	Dr.T.Terasima (NIRS)
16:55	"Malignant cell transformation induced by tritiated water in mouse 10T1/2 cells" Dr.T.Yamaguchi (NIRS)	
18:00	RECEPTION (NIRS)	

Date Time	Subject	Chairmen
Oct.31(Wed)		
*SESSION III.	CHROMOSOMAL EFFECTS OF TRITIUM	
9:30	"Chromosomal effects of tritium on lymphocytes of the teleost, Umbra limi" Dr.I.Suyama (NIRS)	Dr.T.Ishihara (NIRS)
10:00	"Induction of SCE by tritium in the mouse bone marrow" Dr.T.Ikushima (RES Reactor Inst, Kyoto Univ)	
10:20	"Development of the monitoring system for human exposure to tritium" Dr.K.Morimoto (Univ Tokyo)	
10:50	BREAK	
*SESSION IV.	TRITIUM EFFECTS ON THE DEVELOPMENT	
11:00	"UV-sensitivity of the mouse sperm and zygotes fertilized in vitro" Dr.T.Yamada (NIRS)	Dr.T.Nakazawa (Toho Univ)
11:20	"Chromosomal effects of tritium in mouse zygotes fertilized in vitro" Dr.Y.Matsuda (NIRS)	
11:40	Dr.R.A.Pedersen (Univ Calif San Francisco) (cancelled)	
12:10	LUNCH	
13:10	"Tritium effects on the gonads of the aquarium fish, Oryzias latipes. 1) Fecundity and fertility" Dr.Y.Hyodo-Taguchi (NIRS)	Dr.T.Shiroya (Univ Tokyo)
13:30	"Tritium effects on the gonads of the aquarium fish, Oryzias latipes. 2) Histological changes" Dr.H.Etoh (NIRS)	
13:50	"Tritium effects on the mouse ovary: Oocyte killing, fertility loss, and genetic implications" Dr.R.L.Dobson (LLNL)	
14:20	BREAK	
*SESSION V.	TRITIUM EFFECTS ON THE HEMOPOIETIC TISSUE AND INTESTINE	
14:35	"Tritium-induced cell death in the intestinal epithelium of the mouse" Dr.K.Ijiri (Univ Tokyo)	Dr.K.Hirashima (Saitama Med Sch)
15:05	"Induction of micronuclei and some other abnormalities following tritium exposure in the bone marrow of the mouse" Dr.M.Kashima (NIRS)	
15:35	"Further studies on the genetic bone marrow damage and other somatic effects following exposure to low-level tritium" Dr.A.L.Carsten (BNL)	
16:05	"Changes in the hemopoietic stem cells and lymphocyte subsets in humans after exposure to some internal emitters" Dr.I.Nakao (NIRS)	

Date Time	Subject	Chairmen
Nov.1 (Thur)		
*SESSION VI.	ENVIRONMENTAL ASPECTS AND HEALTH PHYSICS OF TRITIUM	
9:30	"Background tritium level throughout Japan" Dr.Y.Takashima (Kyushu Univ)	Dr.M.Sakanoue (Kanazawa Univ)
10:00	"Environmental aspects of tritium released into the atmosphere in the vicinity of nuclear facilities in Japan" Drs.T.Iwakura and Y.Inoue (NIRS)	
10:30	"Environmental tritium transport model. Application to the dose evaluation of members of the public" Dr.C.E.Murphy, Jr (SRL)	
11:00	BREAK	
11:10	"Experience of tritium monitoring for work place and environment in JAERI" Dr.M.Murata (JAERI)	Dr.Y.Yoshida (JAERI)
11:40	"Experience of tritium protection and exposure of workers in JAERI" Dr.J.Akaishi (JAERI)	
12:10	CLOSING Dr.G.D.Duda (US. DOE)	

Contents

Opening Address ---------------------------------- M.Wada --- 1

Welcome Speech ------------------------------- T.Kumatori --- 3

Uptake and metabolism of tritium gas and vapor in the rat
--------------------------------- Y.Ichimasa and Y.Akita --- 5

Tritium metabolism in newborn mice.
--------------------------------- M.Saito and M.R.Ishida --- 19

Studies on the tritium uptake in edible plants and
transfer to the rat. --------------- K.Arai and H.Takeda --- 35

Metabolism of organically bound tritium ------- C.C.Travis --- 50

Microdosimetry of tritium beta particles ----------- A.Ito --- 62

Tritium effects on DNA : Tritium concentration dependency
of RBE in aqueous solution
---------------------------- T.Kada,Y.Sadaie and T.Inoue --- 64

Radiobiological effects of tritiated water on yeast : High
incidence of division anomaly of yeast cells in HTO
containing growth medium --------------- T.Ito and A.Ito --- 75

Analysis at the molecular level of mutations induced by
tritium,X-rays and chemical mutagens ----------- W.R.Lee --- 81

Dose rate effects of mutation induction by tritiated water
in mouse leukemia cells.
------------- N.Nakamura,N.Morikawa,N.Nogawa and S.Okada --- 95

Mutation induction by tritiated water and effects of some

agents in mouse leukemia cells.
---------------------- I.Furuno-Fukushi and H.Matsudaira ---101

Deuterium oxide inhibition of gamma ray dose-rate effects on growing mouse leukemia cells.
------------------------------ A.M.Ueno and H.Matsudaira ---110

Malignant cell transformation induced by tritiated water in golden hamster embryo cells.
--------------------------------- O.Nikaido and F.Suzuki ---127

Malignant cell transformation induced by tritiated water in mouse 10T1/2 cells.
----- T.Yamaguchi,M.Yasukawa,T.Terasima and H.Matsudaira ---136

Chromosomal effects of tritium on lymphocytes of the teleost, Umbra limi.---------------- I.Suyama and H.Etoh ---146

Induction of SCE by tritium in the mouse bone marrow.
------------------- T.Ikushima, R.D.Benz and A.L.Carsten ---157

Development of the monitoring system for human exposure to tritium : Chromosome aberrations in human lymphocytes exposed to HTO ----------------------------- K.Morimoto ---173

UV sensitivity of the mouse sperm and zygotes fertilized in vitro. ----- T.Yamada,Y.Matsuda,H.Ohyama and A.Ohkawa ---182

Chromosomal effects of tritium in mouse zygotes fertilized in vitro. -------------- Y.Matsuda,T.Yamada and I.Tobari ---193

Tritium effects on the gonads of the aquarium fish,Oryzias latipes. 1. Fecundity and Fertility.
----------------------------- Y.Hyodo-Taguchi and H.Etoh ---207

Tritium effects on the gonads of the aquarium fish, Oryzias latipes. 2. Histological changes.
------------------------------ H.Etoh and Y.Hyodo-Taguchi ---221

Tritium effects on the mouse ovary : Oocyte killing, fertility loss, and genetic implications.
---------------------- R.L.Dobson,T.C.Kwan and T.Straume ---231

Tritium-induced cell death in the intestinal epithelium of the mouse ------------------------- K.Ijiri and T.Shiroya ---233

Induction of micronuclei and some other abnormalities in mouse bone marrow following tritium exposure.
---------------------- M.Kashima,H.Joshima and K.Fukutsu ---246

Further studies on the genetic damage to bone marrow and other somatic effects following exposure to low level tritium. -- A.L.Carsten,R.D.Benz,S.L.Commerford,W.Hughes, Y.Ichimasa,T.Ikushima and H.Tezuka ---258

Changes in the hemopietic stem cells and lymphocyte subsets in humans after exposure to some internal emitters. -- I.Nakao,I.Jinnai,M.Bessho,Y.Kawase,M.Ohtani, H.Sugiyama and K.Hirashima ---276

Background tritium levels throughout Japan -- Y.Takashima ---287

Environmental aspects of tritium released into the atmosphere in the vicinity of nuclear facilities in Japan.
----------------------- Y.Inoue,K.Miyamoto and T.Iwakura ---296

Environmental tritium transport model: Application to the dose evaluation for members of the public.
--- C.E.Murphy,Jr ---316

Experience of tritium monitoring for work place and environment in JAERI. ----- M.Murata,K.Obata,T.Ohhata, H.Katagiri,H.Yamamoto, M.Kokubo and Y.Yoshida ---333
Experience of tritium protection and exposure of workers in JAERI ------- J.Akaishi,H.Fukuda,T.Hattori and S.Suga ---346

Opening address

Mr. Masatake Wada

Good morning Gentlemen,

It is our great pleasure to have an opportunity to hold this Workshop with many participants including six specialists from the United States of America. We'd like to express our sincere welcome to all participants.

And now, as you know, nuclear fusion is a very promising energy resource which may contribute greatly to the welfare of human beings in the future. Japan is actively promoting research and development on nuclear fusion, aiming at putting it into practical use.

The energy breakeven plasma condition is expected to be achieved in the latter half of 1980's. For this purpose, JT-60, a tokamak-type energy breakeven plasma testing device, is now under construction in JAERI, aiming its completion in next April.

The development of practical fusion energy is a common, long-term goal of human beings, and it is in our mutual interest to pursue this goal as effectively as possible.

I understand that the study of tritium radiobiology and health physics is one of the most important research item for the safety and public acceptance of Fusion Power Reactors, so I hope this Workshop will be a significant step and contribute to the cooperation on Fusion Research and Development between the United States and Japan.

Finally, I would like to express our sincere gratitude to the key persons', Dr. Goldstein, Dr. Matsudaira and the staff of National Institute of Radiological Sciences who have made a great effort in preparing for this Workshop.

Thank you.

Welcome speech

Dr. Toshiyuki Kumatori

Mr. Chairman, Ladies and Gentlemen,

It is my great pleasure and honour to welcome all of you to this "Workshop on Tritium Radiobiology and Health Physics". This is the second in a series and held under the auspice of the governments of the United States and Japan, as a Cooperation Program in Nuclear Fusion.

At the time of the first workshop in 1981, I briefly described the background and necessities for promotion of tritium research in our country. During the past three years, the situation remained largely unchanged. However, there is a slow but steady progress achieved in fusion research in general. For example, at the Japan Atomic Energy Research Institute in Tokaimura, researches for production and handling of quantities of tritium have been initiated in 1983. A huge facility for fusion experiments has been established there quite recently with many people working under national and international cooperation programs.

The same may apply to tritium radiobiology and health physics. Thanks to the supports of Ministry of Education, and Science and Technology Agency and to the enthusiasm of workers concerned, facilities have been set up in late 1970s in a number of institutions including our own. Collaborative or projected researches have been under way

since early 1980s.

Unlike the achievements on Oak Ridge, Brookhaven, Lawrence Livermore, Mol in Belgium and other institutions, the work carried out in our country until now are certainly small in scale, but I hope, fundamental important researches will be expanded in due course.

Last but not least, may I express my hearty welcome especially to participants from the United States, Dr. Dobson, Dr. Carsten, Dr. Murphy, now our old friends, and Dr. Duda, Dr. Lee and Dr. Travis, our new friends, who have come to Chiba in spite of their heavy schedule.

I said at last meeting that tritium research was in an embryonic stage. But I am confident that, with the help of US participants, two and a half days discussions be concentrated on certain subjects to give birth to a healthy child.

We are now in one of the best seasons of the year. I sincerely hope you would enjoy with the help of Japanese attendants our culture and beautiful scenery during your stay here in Japan. This may promote mutual understanding and deepen the friendship between the United States and Japan.

Thank you for your attention.

NIRS-M-52, pp 5 - 18 ,1985

Uptake and Metabolism of Tritium Gas and Vapor in the Rat

Y.Ichimasa and Y.Akita

Department of Biology
Faculty of Science
Ibaraki University
Mito-shi, 310, Japan

ABSTRACT

Rats were kept in a metabolic cage through which air containing tritiated water vapor was passed at a constant rate. Tritium acitivty in blood and urine increased in proportion to that in air to which the rats were exposed and the absorption rate of tritiated water (the ratio of tritium activity in blood or urine to that in air) was constant at various humidities. When the rats were exposed to continuous inhalation of tritiated water vapor (10.1 pCi/cm^3 air) tritium activity in urine increased progressively with time and attained to about 45 nCi/ml after 72 hours. Tritium activities in various tissues were determined after exposing the rats to tritiated water vapor for various periods.

The tritium gas inhalation apparatus was constructed, which consisted of a tritium gas supply system, an exposure chamber system and a tritium removal system. Rats were kept for 140 min in the metabolic cage of the apparatus through which air containing tritium gas of 10 nCi/cm^3 air was circulated. The tritium activity of the expired water vapor increased almost linearly with exposure time after 20 min of lag time and the total amount of tritiated water was 0.46 nCi/ml after 140 hours.

INTRODUCTION

Tritium is the radioactive isotope of hydrogen. It is stated that tritium gas is not significantly absorbed into the body whereas tritiated water is absorbed and rapidly distributed throughout the body[1). This is based essentially on the experiments by Pinson and Langham[2). They reported that equivalent amount of tritium appeares in the body fluids of rats after exposure when the activity of tritium gas in the ambient air was about 1500 times that of tritiated water vapor. Since then, no reports have been published on the absorption of tritium gas in the body.

The present study was undertaken to confirm the results reported by Pinson and Langham. We designed two experimental apparatus for exposing rats to tritium gas and tritiated water under defined conditions. The rates of uptake of tritium in rat following tritium gas inhalation were compared with those following exposure to tritiated water vapor.

MATERIALS AND METHODS

Animal

Wister strain male rats (9 to 11 week-old) were used. They were fed standard diet and given water *ad libitum* and were maintained in a room or metabolic cage with controlled air and temperature.

Tritium gas and tritiated water

Tritium gas with a specific activity of 200 mCi/cm^3 was diluted to an appropriate concentration with dried air. Tritium gas diluted with air(10 nCi/cm^3) was introduced into a microcombustion tube where tritium gas was converted to tritiated water by means of 0.5 % Pt-aluminium pellet (Nippon Engelhard Co.) at 350 °C. Tritiated water was absorbed in silica gel. This silica gel was used as a source of tritiated water vapor supply.

Analysis of tritium activity in body fluids and tissues

Rats were killed at various time intervals after tritium inhalation and tissue and blood samples were collected. A part of each tissue or blood sample was weighed and immediately analysed for total tritium activity. Another part of each sample was thoroughly lyophilized for analysis of tissue-bound tritium activity. Tritium activity was determined by a liquid scintillation spectrometer after combustion in an oxidizer (Model 306 Automatic Tri-Carb Sample Oxidizer, Packard Instrument Co.).

Exposure of rats to tritiated water vapor

Rats were exposed to tritiated water vapor in the one-way inhalation apparatus illustrated in Fig. 1. Air was pumped through the inlet tube into gas flow meter and to a dried silica gel bottle and then a -60°C cold trap. The dried air from the

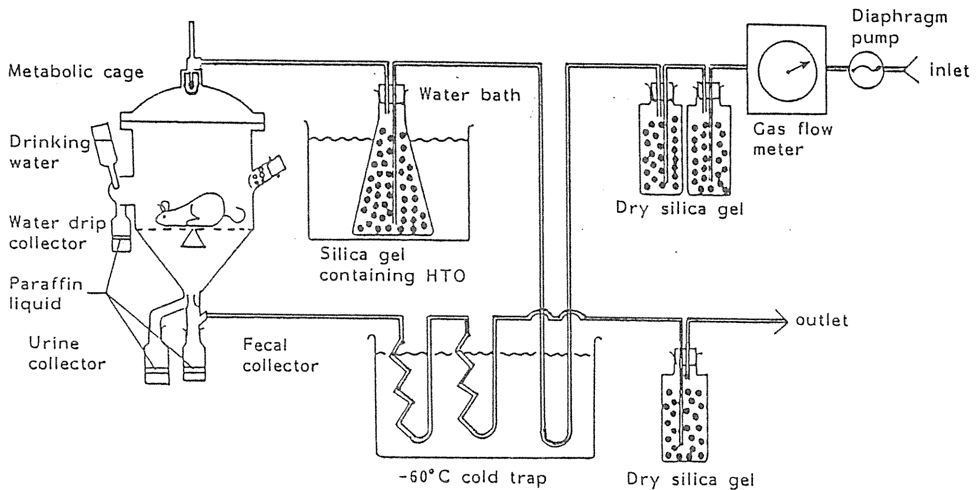


Fig. 1. Experimental apparatus for exposing rats to tritiated water (HTO).

cold trap was introduced into the bottle filled with silica gel containing tritiated water and humidified with tritiated water vapor released from the silica gel. The radioactive air was then introduced into the metabolic cage connected through a -60 °C cold trap to a bottle filled with dried silica gel. To prevent evaporation of tritiated water, an overlay of liquid paraffin was used in each collector(a water drip collector, urine collector and fecal collector). Absolute humidity and tritium activity in the air was calculated by measuring weight and radioactivity of the trapped water collected by the cold trap.

Exposure of rats to tritium gas

The apparatus used for total body exposure was a closed system in which the gas was continuously circulated around the respiring animal while a constant tritium level and normal atmospheric pressure were maintained (Fig. 2) The exposure was started by introducing tritium gas from the bulb (A) into the metabolic cage. The gas was then driven by circulating air through a -60 ℃ cold trap to remove any trace of tritiated water. The air

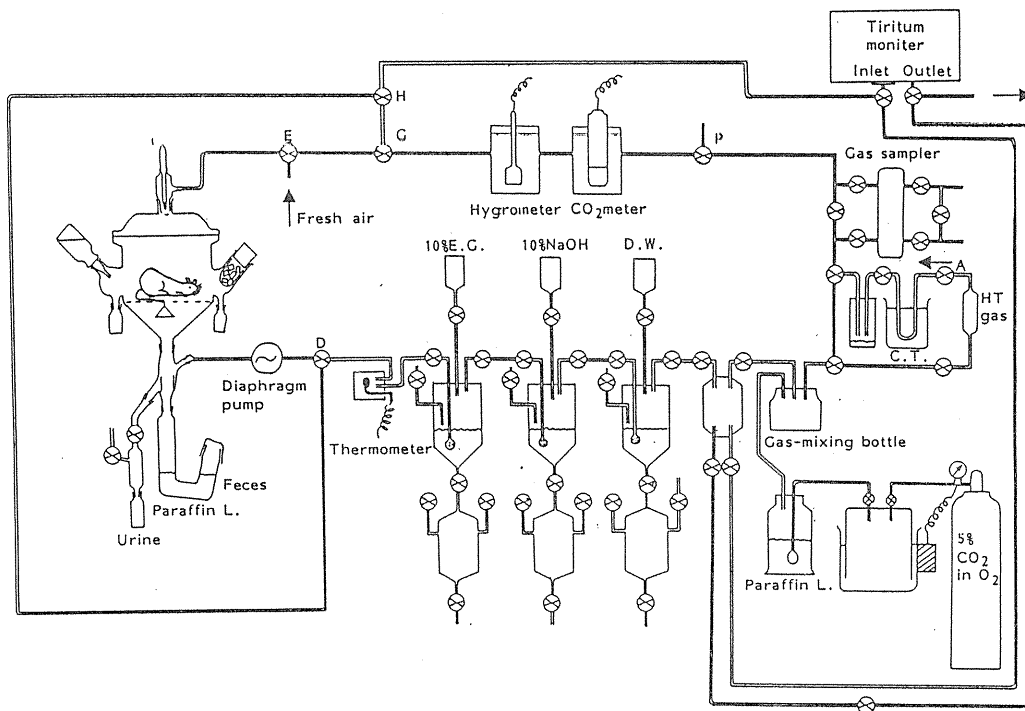


Fig. 2. Experimental apparatus for exposing rats to inhalation of tritium gas (HT).

containing tritium gas was then pumped by an air-tight circulating diaphragm pump through successively three gas washing bottles each filled with 10 % ethylene glycol, 10 % NaOH solution and distilled water, (to absorb HTO, CO_2 and to wash the air) respectively. The air was then introduced into a tritium monitor (Model MGR-108C, Aloka Co.) for recording tritium concentration in the circulating air. The ambient pressure was maintained at a constant level, while the animal was breathing, by introducing carbon dioxide and oxygen into the system. Just before the end of exposure 78 cm^3 of atmosphere was collected through a gas sampler and passed over Hopcalite II (Nakarai Chemicals Co.) at 600 °C. Then, the metabolic cage was flushed out with fresh air for 15 min so that the recording device indicated no significant amount of tritium remaining. The exhausted air containing tritium gas was passed over 0.5 % Pt-alminium pellet at 350 °C to

convert all the tritium to tritiated water which was then absorbed by silica gel. After that the rats were removed and sacrificed.

RESULTS

1. Tritium intake by rats exposed to tritiated water vapor

To administer a constant amount of tritiated water vapor to rats for a long period, a tritiated water vapor inhalation apparatus was designed (Fig. 1). The amount of tritiated water vapor in the air in the apparatus was changed by keeping the silica gel bottle, which contained tritiated water, at various temperature. As shown in Fig. 3, the amount of tritiated water vapor in the air was dependent on the incubation temperature of the silica gel and was almost constant for periods of 140 or 220 hours if temperature of the silica gel was kept constant.
Figure 4 shows the time required to get a constant humidity in the inhalation apparatus when rats previously kept under different humidities were transferred into the metabolic cage of

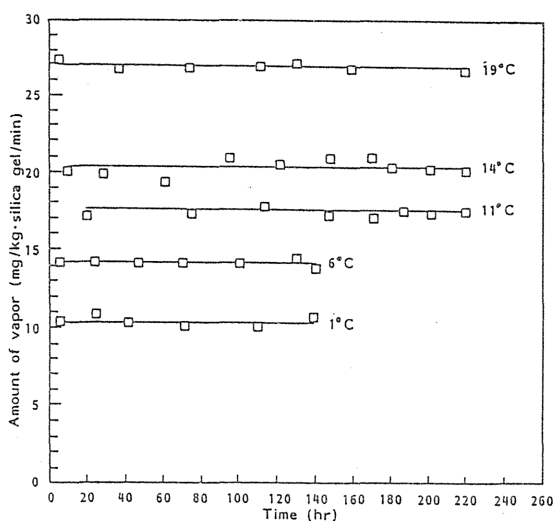


Fig. 3. Amount of vapor released from silica gel at various temperatures. Flow rate of air was 3000 cm^3/min.

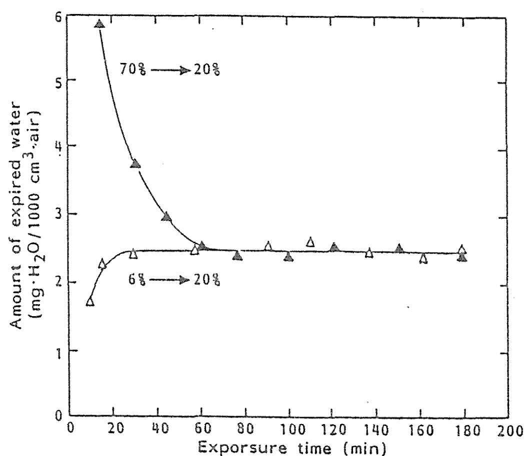


Fig. 4. Amount of expired water from rats exposed to the air of 20 % humidity. Rats previously kept at 70 % or 6 % humidity were transferred to the metabolic cage of 20 % humidity (4.1 mg H_2O/1000 cm^3 air).

the apparatus where a constant flow (3000 cm^3/min) of air containing tritiated water was maintained. As shown in Fig. 4, when a rat previously kept under 70 % humidity was transferred to the metabolic cage of 20 % humidity, it took at least 60 min to get a constant humidity. So, it is important that rats be kept under the same humidity as the experiment for more than one hour before use.

Figure 5 shows the changes in the tritium activity in blood and urine as a function of the activity in air. Rats were exposed to tritiated water vapor for 2 hours at 23°C. Flow rate of air was 3000 cm^3/min. A period of two hours was found enough essential equilibrium was established between the ambient air and fluid of the rat[3). The tritium activity in blood and urine (Y) tended to be a linear function of that in the air (X) and could be fitted to folowing equations:

Y (nCi/ml) = 0.0842X (pCi/cm^3) + 0.1354, for blood with a correlation coeficient of 0.982, and

Y (nCi/ml) = 0.0609X (pCi/cm^3) + 0.0816, for urine with a correlation coefficient of 0.938.

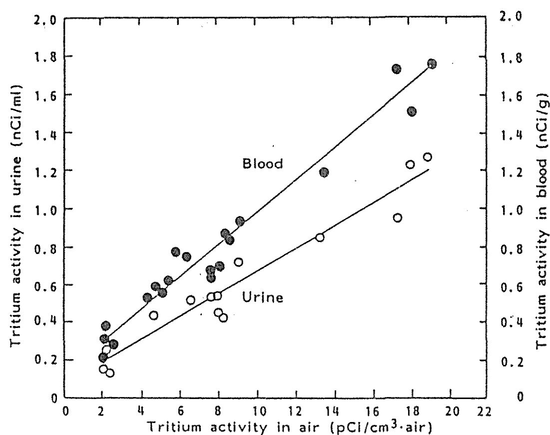


Fig. 5. Tritium activity in blood (●) and urine (○) as a function of tritium activity in air. Rats were exposed to tritiated water vapor (4-6 mg H_2O/1000 cm^3 air) for 2 hours at 23°C.

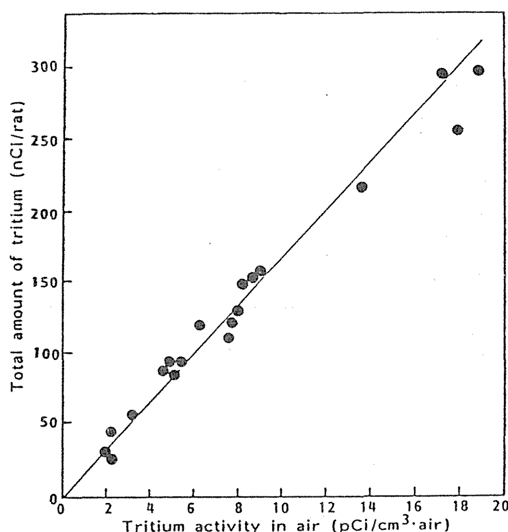


Fig. 6. Total amount of tritium in rat as a function of tritium activity in air.

Fig. 6 shows the changes in the total amount of tritium activity in the body of rat as a function of the concentration in the air. The total tritium activity in rats (A) was calculated from the dilution volume (63 % of the body weight) and the body weight using the following formula:

A = tritium activity in blood (nCi/ml) x body weight x 0.63

The dilution volume was determined by measuring the changes in the tritium activity in venous blood from 30 min to 3 hours after intraperitoneal injection of tritiated water. As shown in Fig. 6, the total tritium activity in the body of a rat was proportional to the tritium activity in the air.

The changes in the relative tritium activity in blood and urine expressed in absorption rate as a function of humidity in air are shown in Fig. 7. The absorption rate of tritiated water, i.e., the ratio of the activity in blood (nCi/g) or urine (nCi/ml) to that in air (nCi/cm^3), was almost independent on the absolute humidity in air and was 106 ± 12 for blood and 69 ± 12 for urine after 2 hour exposure, respectively. The ratio for blood was 55 % higher than that for urine. These results show that when rats were exposed to tritiated water vapor in this

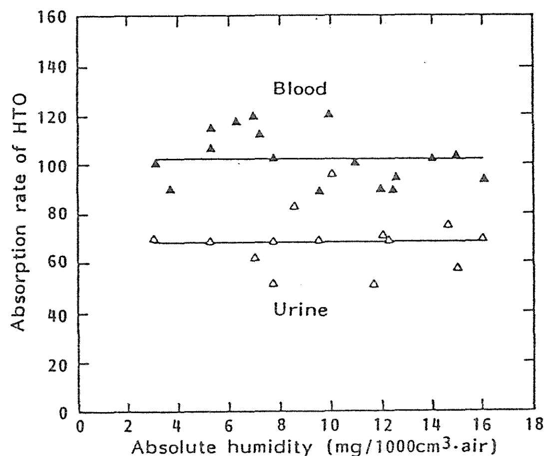


Fig. 7.

Absorption of tritiated water vapor into blood (▲) and urine (△) at various humidities.

$$\text{Absorption rate} = \frac{\text{Tritium activity in blood } (\mu Ci/g) \text{ or urine } (\mu Ci/ml)}{\text{Tritium activity in air } (\mu Ci/cm^3)}$$

one-way system uptake of tritium by rats was quantitative and dependent on the amount of tritiated water in air.

Figure 8 shows the changes of tritium activity in urine as a function of exposure time. Rats were exposed to tritiated water vapor (10.1 pCi/cm^3 air) for periods up to 140 hours. The tritium activity in urine progressively increased with time and attained to a level of about 45 nCi/ml after 72 hours. Pinson and Langham[2)] have reported a value of 120 nCi/ml urine when rats were exposed to tritiated water vapor (26 pCi/cm^3 air) for 72 hours. The absorption rates of tritiated water vapor calculated from the data by Pinson and Langham agree well with those calculated from the present data.

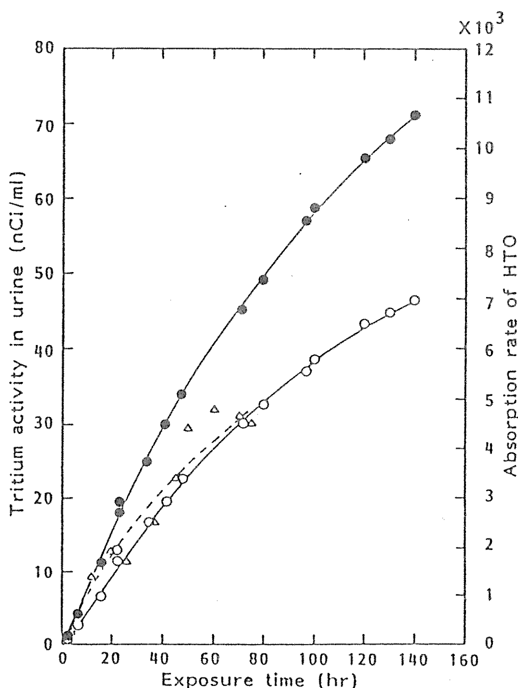


Fig. 8. Tritium activity in urine during exposure to tritiated water vapor (10.1 pCi/cm^3 air) and comparison of our results with those obtained by Pinson and Langham (1957). Absolute humidity, 8 mg/1000 cm^3 air. ●, tritium activity in urine; ○, absorption rate of HTO; △, absorption rate of HTO (data by Pinson and Langham[2)], normalized to present experimental conditions).

Figure 9 shows the tritium activity in blood and various tissues at different times after commencement of continuous exposure to titiated water vapor. During the first 3 hours the tritium activity in the lung tended to be somewhat higher than that in the other tissues tested but no significant differences were detected among the latter tissues. After 35 hours on, however, tritium activity in adipose tissue did not rise much and remained at about one-tenth of that of liver or lung at 140 hours.

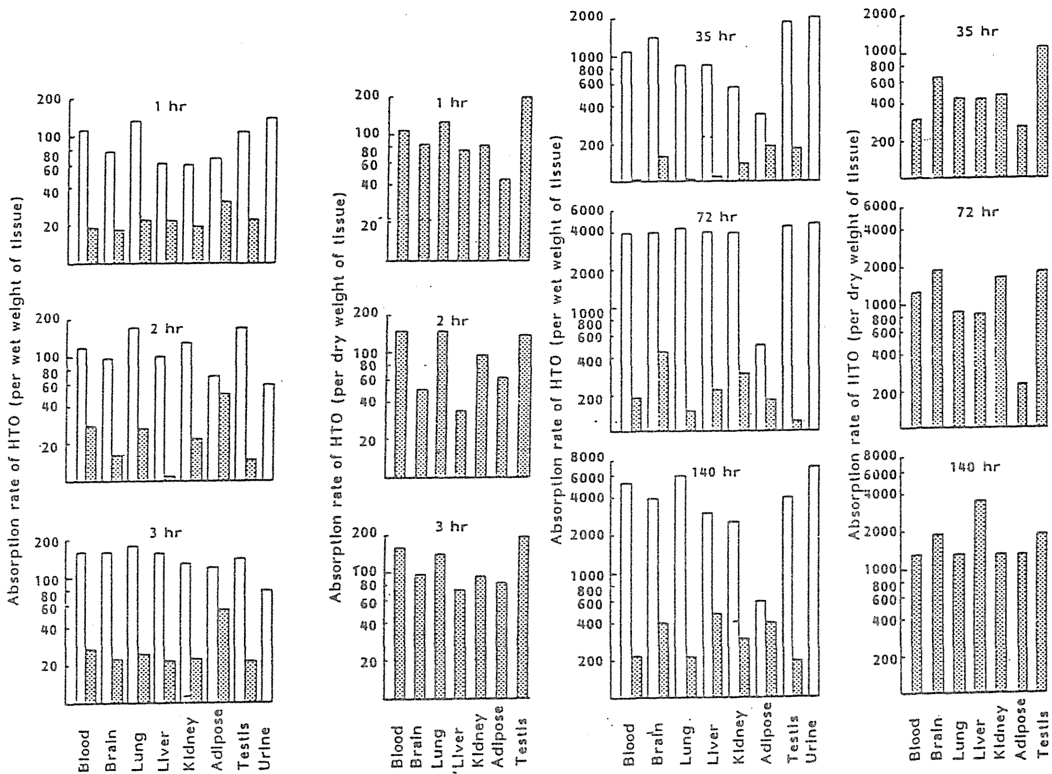


Fig. 9. Tritium activity in blood, various tissues and urine during tritiated water vapor. Open bar, total tritium activity; dotted bar, tissue-bound tritium activity.

2. Tritium uptake in rats exposed to tritium gas

Rats were kept for 140 min in the metabolic cage of the tritium gas inhalation apparatus (Fig. 2) through which air containing tritium gas (10 nCi/cm^3) was circulated. The expired water vapor was measured for tritium actiivty. As shown in Fig. 10,

the tritium activity of the expired water vapor increased almost linearly with exposure time after 20 min of lag and the total amount of tritium in expired water attained a value of 0.46 nCi/ml after 140 hours.

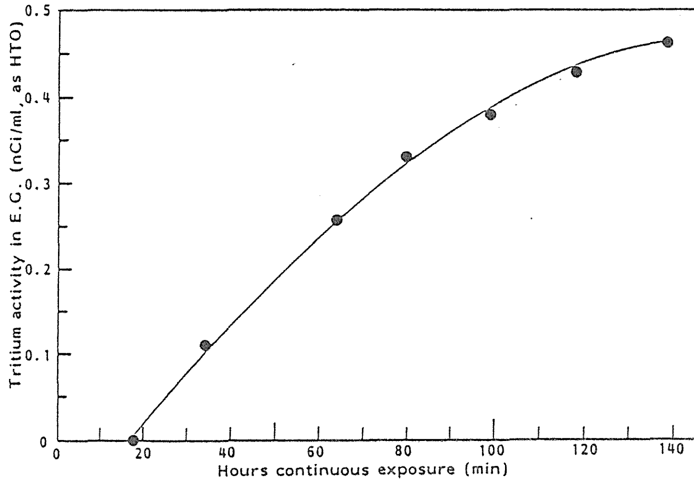


Fig. 10. Tritium activity (as HTO) in ethylene glycol (E.G.) during inhalation of air containing 10 nCi/cm^3 of tritium gas. Tritium activity in E.G. represents that in expired water vapor.

Table 1 summarizes the rate of incorporation of tritium in blood of rats after exposure to tritium gas in comparison with tritiated water vapor. The rate of incorporation from tritium gas was one-half of that from tritiated water even when the specific activity of tritium gas in the air was 1000 times that of tritiated water, this means that tritium gas is taken up as much as 2000 times less than tritiated water vapor by the rat.

Table 1. Intake of tritium from tritiated water vapor and tritium gas into rat blood

Tritium	Inhalation time (min)	Tritium activity in air (pCi/cm^3)	Tritium activity in rat blood (nCi/g)
HTO vapor	140	10.1	1.37
HT gas	137	10000	0.72

DISCUSSION

Aune *et al.*[4] developed a filter with silica gel to remove tritiated water vapor from the effluent air of a glove box. We found that silica gel containing a considerable amount of tritiated water released tritiated water vapor continuously for a long period of time and the amount of tritiated water vapor was dependent on the incubation temperature of the silica gel. This prompted us to use the silica gel as a source of tritiated water vapor supply in the experimental apparatus designed for exposing rats to tritiated water (Fig. 1).

As shown in Fig.7, the tritium activity in urine was lower than that in blood after 2 hour exposure to tritiated water vapor. The rats had been forced to urinate just before the experiments. Therefore, the possibility of dilution of urinary tritium by urine remaining in the urinary bladder could be excluded. At later period of exposure the rate of incorporation of the tritium into urine increased, often to a level slightly higher than that in blood (Fig. 9) and after 385 hours the tritium activity in urine was almost the same as that in blood (data not shown). Pietrzak-Flies *et al.*[5] made similar observations: the tritium activity in urine of rabbits given tritium in drinking water daily was the same level as that in blood after 336 hours.

When rats were exposed continuously to tritiated water vapor (10.1 pCi/cm^3 air) in the inhalation apparatus of one-way system under a constant flow of air (3000 cm^3/min) and relative humidity of 40 %, the tritium activity in urine increased with time after exposure and attained a level of 45 nCi/ml after 72 hours (Fig. 8). Pinson and Langham[2] have reported a value of 120 nCi/ml

urine/72 hours when rats were exposed to tritiated water vapor (26 pCi/cm^3 air) in a closed system of approximately 30 liters in volume under water saturated atmosphere. Both data are in good agreement with each other. This also supports our results in that the rate of incorporation of tritium into body fluids was dependent on the total tritium activity, not on the humidity of the air (Fig. 7). The tritium activity in unit volume urine was about 6.4 % of that of tritiated water in the air after 140 hours of exposure. The shape of uptake curve suggests that after very long periods of exposure, the tritium level in urine approaches that in the air.

When rats were exposed to tritium gas, there was a lag time of 20 minutes before tritium activity appeared in the expired water vapor (Fig. 10). This may represent the time during which the inspired tritium gas is transferred to the intestine, and oxidized by bacteria[6,7] to tritiated water, which, in turn, circulared and expired.

Tritium activity in blood of rat after exposure to tritium gas was about one-half of that resulting from exposure to tritiated water vapor even when the specific activity of tritium gas was 1000 times that of tritiated water. Considering the relation of tritium activity in blood to that in the ambient air (Fig. 5), one can say that equivalent amount of tritium appears in blood when the activity of tritium gas in air is 2000 times that of tritiated water vapor.

AKNOWLEDGEMENT

We are grateful to Dr. H. Matsudaira for critical reading of the manuscript. This work was supported by research grants from

the Ministry of Education, Science and Culture of Japan (Nos 58050007 and 59050009).

REFERENCES

1. NCRP Report 65, Management of persons accidentally contaminated with radionuclides. NCRP, Washington (1980).

2. E. A. Pinson and W. H. Langham, Physiology and toxicology of tritium in man. J. Appl. Physiol. 10, 108-126 (1957).

3. Y. Ichimasa and Y. Akita, Water metabolism and modification of tritium excretion in the rat. NIRS-M-41, 205-218 (1982).

4. R. G. Aune, H. P. Cantelow and R. L. Boltin, Practical control of tritiated water vapor by silica gel and of tritium gas by the use of a purging device. In "Tritium" Ed. A. A. Moghissi and M. W. Carter, Messenger Graphics, Nevada (1973).

5. Z. Pietrzak-Flis, I. Radwan and L. Indika, Tritium in rabbits after ingestion of freeze-dried tritiated food and tritiated water. Radiat. Res. 76, 420-428 (1978).

6. G. N. Smith and R. O. Marshall, Fixation of molecular tritium by bacterial suspensions. Arch. Biochem. and Biophys. 39, 395 -405 (1952).

7. G. N. Smith, R. J. Emerson, L. A. Temple and T. W. Galbraith, The oxidation of molecular tritium in mammals. Arch. Biochem. 46, 22-30 (1953).

NIRS-M-52, pp 19 - 34 , 1985

Tritium Metabolism in Newborn Mice

M.Saito and M.R.Ishida

Research Reactor Institute
Kyoto University
Kumatori-cho, Sennan-gun, 590-04 Osaka, Japan

Abstract

Studies on tritium metabolism and dosimetry were carried out in two aspects in newborn mice which received tritium during their suckling stage.

First, a computer simulation was done to estimate the dose-modifying factor due to localization of the DNA-bound tritium to cell nucleus. The results suggested that a factor of about between 2 and 6 should be multiplied to the dose calculated for an assumed homogeneous distribution of tritium in the whole cell.

Second, suckling mice received tritium orally from their mothers which were supplied with tritiated water. The offsprings were sacrificed at various stages after weaning and the contributions of various molecular components to the total remaining tritium in the several organs were determined. Tritium was most strongly retained by lipid and protein components. At 31 weeks after birth, the highest concentration of remaining tritium was found in the brain where tritium is predominantly retained by the lipid component. By using the metabolic data, the contribution of the

organically bound tritium to the accumulated dose was estimated to be 15 - 40 % varying among various organs ; the largest value was found for brain. This result suggests that the absorbed dose due to the organically bound tritium is of significance for assessment of biological effects of tritium intake if an adequate localization factor is taken into consideration for each molecular component.

Introduction

Increasing need for finding a new energy source as a substitute for fossil fuels urges development of nuclear fusion reactors for practical use. One of problems in the development of the fusion reactor is the assessment of environmental influence of tritium released from fusion power plants during routine operation or accident (1).

Several spects of the toxicity of the various chemical forms tritium have been studied. One is the estimation of the hazards of tritium exposure in fetal and neonatal animals (2-7). In most organs tritium is excreted rapidly as free water component (8,9). However, in rapidly proliferating organs, the incorporated tritium is fixed in part as organically bound tritium.

In a previous study (9) in which pregnant mice were supplied with ^{3}H-thymidine containing water, it was found that tritium is strongly retained by the organs, like lung, kidney and brain with little or low proliferating activity after birth. In that experiment, more than 80 % of the total tritium incorporated was found to be converted to the acid-soluble component through metabolic process in the mothers and embryos. On the other hand, it was noticed that tritium is strongly retained in the lipid component of the brain. This high organ-specifity of dose accumulation for DNA-bound tritium is due possibly to organ-specific turnover rate of DNA. These findings stimulated us to carry out further experiments using tritiated

water as an initial tritium source which is supplied to mother mice in lactation.

To evaluate the extent of biological hazards from a given internal exposure to tritium β-rays, establishment of proper dosimetry is prerequisite for the estimation of the accumulated dose in various organs. For tritium existing as the free-water in the cell the dosimetry is rather simple compared with that of organically bound tritium. For the free-water tritium, a homogeneous distribution may generally be assumed. The ICRP recommendations, however, consider in some cases local heterogeneous distribution,in particular of organically bound tritium (10). The localization of tritium in the cells or organs of young animals which received tritiated water is then of significance for estimation of the dose accumulated in critical intracellular substructures. Contribution to the total dose of the organically bound tritium after intake of tritiated water has been estimated to be of the order of 10 % (11). The present work was aimed at re-evaluating this value in suckling mice by taking into account heterogeneous distribution of tritium in the cells.

Materials and methods

Animals. Conventional DDY mice originally obtained from Awadzu Jikkendoubutsu Co., Osaka and colony-bred in our laboratory were used. The animals administered with tritiated water or other tritium compounds were kept in an isolated cabinet system designed for breeding tritium-given small animals. Eight week-old mice were mated for 1 day. After mating, female mice were isolated individually. At noon of the day of delivery, the drinking water for the mother mice was changed to tritium containing water. Tritium administration continued until 3 weeks after delivery. Tritium concentration in drinking water was 370 kBq/ml throughout the experiments.

Determination of tritium retention rate. At various ages, the male offsprings were sacrificed and tritium contents in various organs and tissue fractions such as acid-soluble, lipid, RNA, DNA and protein fractions were determined. The details were described in a previous paper (9).

Determination of average nucleus diameter. The average nucleus diameter was determined by photographing the cell nuclei after staining with a DNA-specific fluorescent dye DAPI (4',6'-diamidino-2-phenylindole) (12) in an isotonic solution under a Nikon fluorescence microscope. The lengths of the major and minor axes of the cell nuclei were determined from the photographs and the mean length of the both axes of individual nucleus was used as the average diameter. The detail of this method was reported previously (13).

Results

The absorbed dose to cell nucleus from DNA-bound tritium is a function of the average nucleus diameter, DNA content of cell nucleus and specific activity of nuclear DNA. The average absorbed dose per disintegration in a cell nucleus was calculated by using a formula given by Tägder and Sheuermann (14). The dose rate in a model nucleus was obtained by Monte Carlo simulation of tritium distribution in a cell nucleus. Such calculation enabled us to estimate the extent of dose reduction due to the escape of tritium beta-rays from the edge of cell nucleus under various conditions. Fig.1 shows the calculated doses for various shapes of cell nuclei of the same volume under the assumption that cell nuclei are ellipsoidal and contain the same amount of DNA-bound tritium. For each curve the volume of a model nucleus was fixed and the ratio of the length of the major axis over the minor (denoted as A and B respectively) was varied. From microscopic

observations, the value of A/B was found to be about between 1 and 5 ; the largest value was found for heart cells. As seen in Table 1, the average diameter of cell nucleus in the examined organs exceeds 5 micrometers. Thus it is suggested from Fig.1 that the dose modification due to the variation of the nucleus shape is less than 5 % of the dose for the spherical nucleus. A slight modification is necessary for the shape of cell nucleus only in an extreme case that the value of A/B deviates considerably from the unity. The dependence of accumulated dose on the diameter of cell nucleus was postulated under the assumption that all cells contain the same quantity of nuclear DNA as spherical cell nuclei do. In most organs examined, the shape of cell nucleus was ellipsoidal and actually could be treated as spherical.

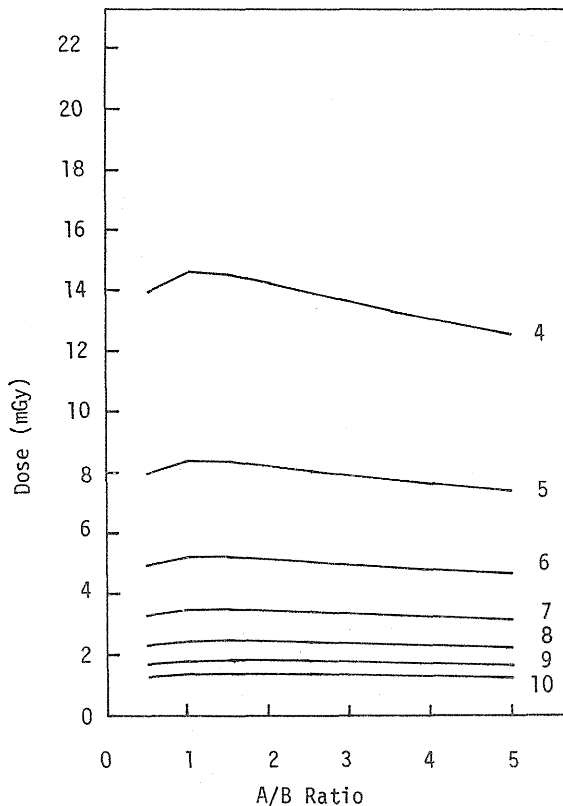


Fig.1 Average absorbed dose per disintegration in model cell nuclei with various shapes. The shape of the cell nuclei is assumed to be spheroidal. The values given on the righthand side of the curves are diameters of the cell nucleus at the extreme case that the cell nucleus is spherical.

Table 1. Average diameter and average DNA content of cell nuceleus in various organs of mouse

Organ	Average diameter of cell nucleus (μm)	Average DNA content of cell nucleus (pg.nucleus^{-1})
spleen	5.6 ± 0.8	9.4 ± 1.5
kidney	6.8 ± 1.3	6.0 ± 0.3
brain	8.5 ± 2.2	7.0 ± 1.3
intestine	8.7 ± 1.1	(7.0)
liver	8.1 ± 1.4	11.9 ± 2.1
heart	(8.0)	(7.0)
lung	(8.0)	(7.0)

The values in the parentheses are those assumed for qualitative discussion.

The extent of the reduction of the energy deposition in cell nucleus due to the energy loss in cytoplasm was about 30 and 20 % for spleen and intestine respectively. This "edge effect" was taken into consideration for the calculation of the curves in Fig.2 which depicts changes in the calculated absorbed dose per disintegration of DNA-bound tritium in a cell nucleus as a function of its diameter. The figure shows that the absorbed dose due to DNA-bound tritium increases with a decreasing mean nucleus diameter. For instance, the absorbed dose in the spleen nucleus is about three times

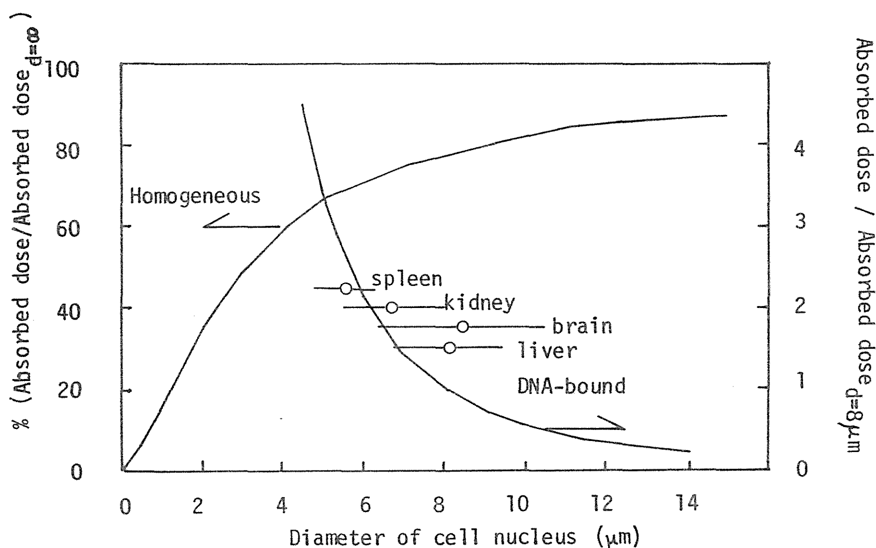


Fig.2 Average absorbed dose per disintegration of DNA-bound tritium and that for tritium when distributed homogeneously in the cell nucleus.

higher than that in the intestine.

The above result was applied for demonstrating the organ-specificity of dose accumulation due to DNA-bound tritium in the newborn mice that received ^{3}H-thymidine transplacentally. Table 2 shows that the accumulated dose in various types of cell nuclei for the period from birth to 4 weeks. For comparison, the accumulated dose was calculated under an assumption that DNA-bound tritium distributes homogeneously in the cells. In this case, values of the dose modifying factor resulting from the tritium localization were about between 2 and 6 ; the smallest was found for the intestine while

Table 2. Accumulated dose in cell nuclei of newborn mice born to mother mice supplied with ^{3}H-thymidine during pregnancy

Organ	Average absorbed dose (mGy)	Average total disintegrations (dis./ng DNA)	Accumulated dose for 4 weeks after birth (mGy)		Localization factor[b]
			DNA-bound	Homogeneous[a]	
Spleen	6.07	31.2	1.78	0.55	3.2
liver	2.26	43.8	1.18	0.23	5.1
intestine	1.86	39.8	0.52[c]	0.34	1.5[c]
lung	2.33[c]	67.5	1.10[c]	0.58	1.9[c]
kidney	3.64	39.8	0.87	0.29	3.0
heart	2.33[c]	109.9	1.79[c]	0.44	4.9[c]
brain	1.98	48.9	0.68	0.11	6.2

a Homogeneous distribution was assumed for the DNA-bound tritium in whole organ.

b The ratio of the accumulated dose for DNA-bound tritium of that calculated for the assumed homogeneous distribution.

c Calculated by using the values given in Table 1 for the assumed average diameter and/or DNA content of cell nucleus.

the largest was for the brain.

Plot against time of total tritium activity per gram tissue in various organs of the mice which obtained tritium from their mothers during suckling stage exhibited retention curves with two major exponential components (Fig.3). The biological hal-lives corresponding to the two components can be roughly estimated from these curves (Table 4). With exception for the case of brain, the value of biological half-life for the

first component was between 2.4 and 2.9 days. In most cases with exception for heart, the second component had half-lives between 110 and 160 days. To clarify to which molecular species the two major components of tritium retention can be attributed, the percent contributions of various molecular components to the total tritium activity were calculated (Fig.4). Fig.5 shows an example of tritium retention curves for individual molecular component, for the brain. Similar curves were obtained for the acid soluble fraction of other organs. It is clear from Figs.4 and 5 that the acid soluble component is the rate limiting factor of tritium retention at the initial stage of animal growth. Our previous results showed that the tritium activity in the acid-soluble component exists predominantly as free water (9). Thus the value of the biological half-life for the first component of tritium retention can be regarded as that for the free water component of remaining tritium.

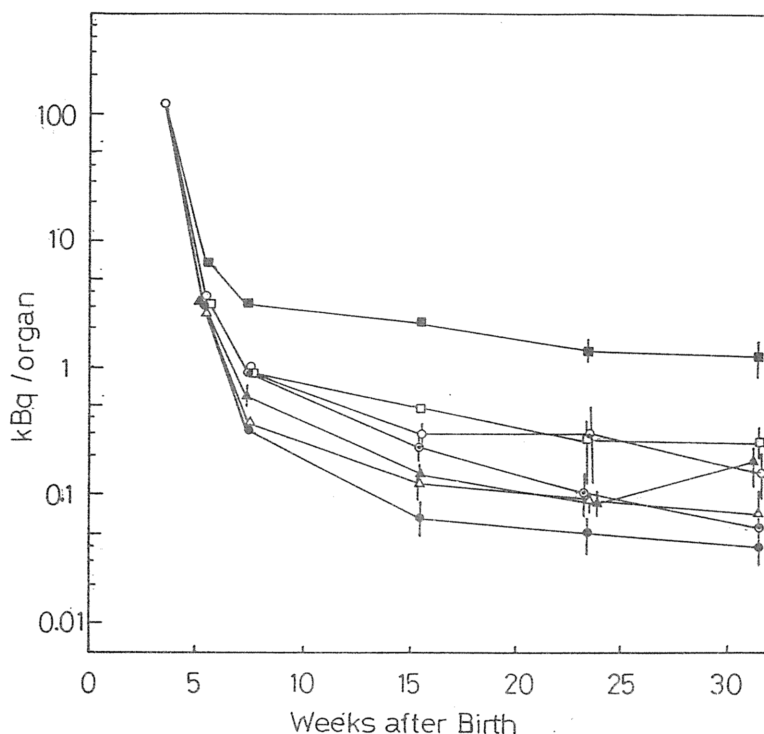


Fig.3 Total remaining tritium activity per gram tissue.
(○), spleen ; (●), liver ; (△), intestine ;
(□), lung ; (▲), kidney ; (⊙), heart ; (■), brain.

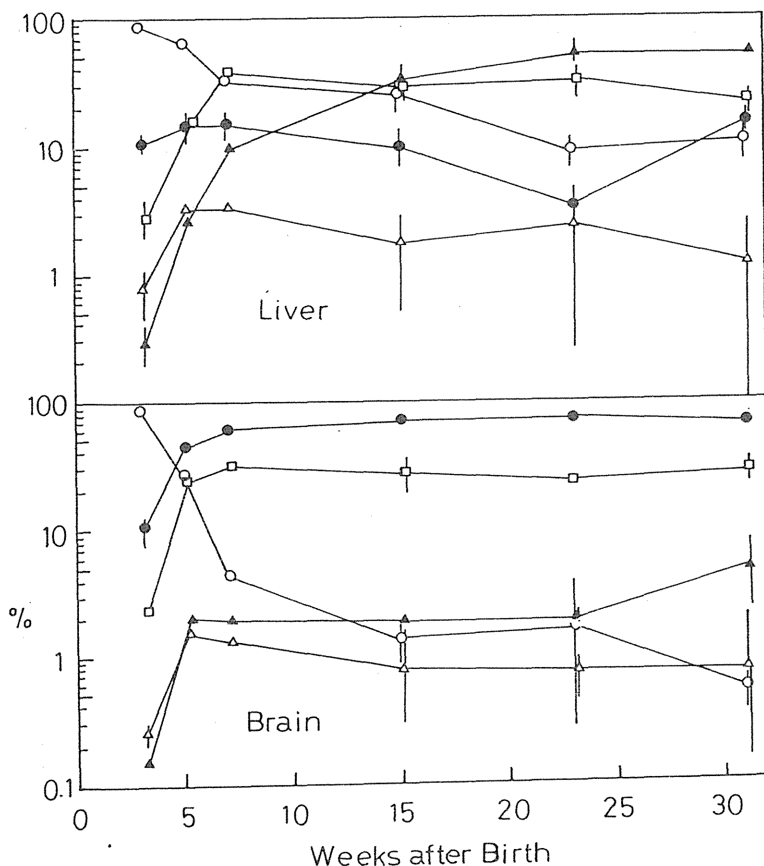


Fig.4 Percent contribution of various molecular components to total remaining tritium activity.
(○), acid soluble component ; (●), lipid ;
(△), RNA ; (▲), DNA ; (□), protein.

Among the five molecular components examined here, only the acid-soluble component contained tritium activity for which the percent contribution decreased with further animal growth after the tritium source was removed. At 3 weeks after birth, the patterns of tritium distribution to various molecular components in various organs were similar each other while they tended to be organ-specific after a long-term experiment. In the liver the DNA component became the major determining factor of remaining tritium activity while in the brain, the lipid component. It is noted from Fig.3 that tritium is most strongly retained in the brain. This is due to the high tritium retention in the lipid component of the brain of the newborn mice. At the

3-week stage, 5 - 12 % of the total tritium activity found in the various organs was due to the tritium in the lipid component. On the other hand, the percent contribution of the lipid component varied considerably among different organs. For example, in the liver, kidney and brain, the lipid component contributed up to 15, 13 and 67% of the total tritium activity,

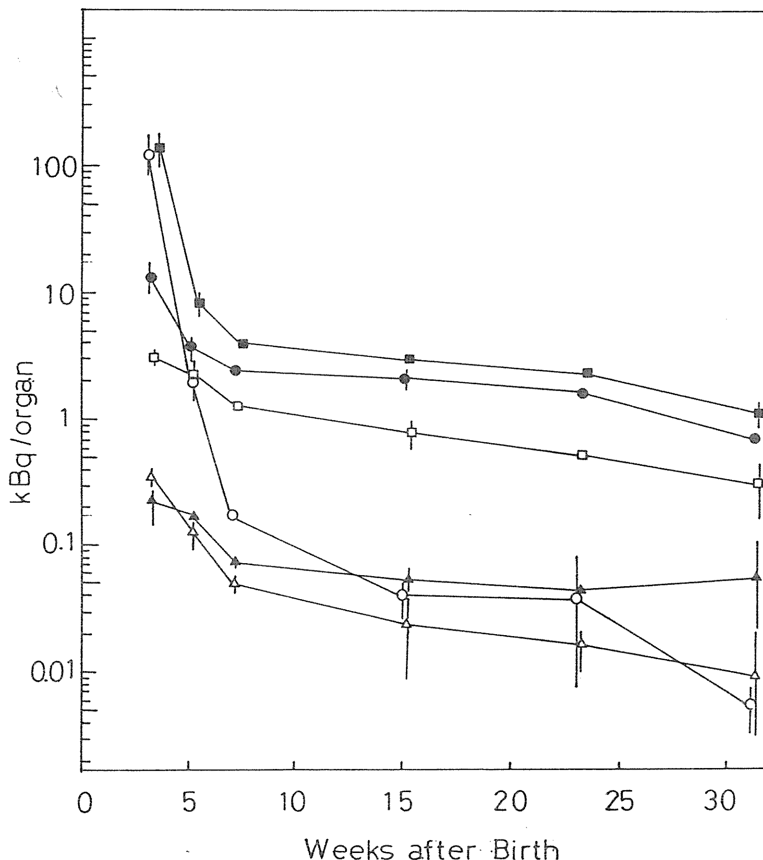


Fig.5 Tritium retention curves for various molecular components in the brain. (○), acid soluble component ; (●), lipid ; (△), RNA ; (▲), DNA ; (□), protein ; (■), total tritium activity.

respectively.

In a long-term exposure to an internal tritium source, the molecular components which dominate residual tritium activity are determining factors of dose accumulation. This is shown in Table 3. The contribution of

various molecular components to the total accumulated dose was calculated by using the metabolic data with a method similar to that given in a previous report (13). Table 3 also shows the dose due to DNA-bound tritium when the tritium localization factors are taken into account for various organs. Under an assumed homogeneous distribution of tritium, the residual tritium in RNA component is unimportant in determining the dose due to organically bound tritium. The values of percent contribution of the lipid component in various organs are between 5.9 and 27.4 ; the lowest value was found for the spleen and the highest for the brain. The percent contribution of the protein component was also organ-specific ; the lowest (3.0 %) was seen for the intestine and the highest (9.7 %) for the brain.

Table 3. Contribution of various molecular components to the accumulated dose for the period between 3 and 31 weeks after birth. Dose unit in mGy.

Organ	Acid soluble[a]	Lipid[a]	RNA[a]	DNA[a]	DNA[b]	Protein[a]	Total[a]
spleen	6.16	0.43	0.09	0.21	0.66	0.43	7.31
liver	6.01	0.78	0.07	0.05	0.27	0.29	7.19
intestine	6.53	0.96	0.07	0.09	0.13	0.24	7.88
lung	5.79	0.56	0.12	0.17	0.31	0.57	7.21
kidney	5.92	0.64	0.05	0.11	0.33	0.34	7.06
heart	5.79	0.52	0.07	0.08	0.37	0.54	7.01
brain	5.91	2.72	0.05	0.08	0.52	0.97	9.92

a Homogeneous distribution was assumed for tritium in whole organ

b Localization of the DNA-bound tritium to cell nucleus was taken into consideration.

Table 4 Biological half-life of tritium in newborn mouse

Organ	First component (days)	Second component (days)
spleen	2.8	118
liver	2.6	159
intestine	2.4	148
lung	2.8	124
kidney	2.6	-
heart	2.9	54
brain	3.3	130

Discussion

The computer simulation can be applied for organically bound tritium if the information is available on the microscopic distribution of molecular components such as protein and lipid. Tritium in the components of high turnover rate will be rather quickly removed from an organ compared with that bound to the molecular components of low turnover rate. Tritium in the protein and lipid molecules of the cytoskelton or nuclear skelton probably represents the latter. At long times after exposure, organically bound tritium observed will be on such stable subcellular structures. To estimate the accumulated dose for a given subcellular compartment, the average track length of tritium beta-rays in the target must be determined experimentally. As found for the DNA-bound tritium, the dose modifying factors for other molecular components will be organ-specific reflecting the variety of subcellular structures. In the case of DNA-bound tritium, the process to obtain a localization factor is rather simplified since the distribution of tritium is restricted to cell nucleus. To find the above modifying factors for other molecular components such as protein and lipid,determination of the subcellular distribution pattern of these molecules in the cell and organ is needed. The present work showed that the organically bound tritium is strongly retained in the mouse brain where tritium is bound to the lipid component. The distribution pattern for the lipid component enabled us to calculate the dose modifying factor for the lipid-bound tritium in brain. A work on this line is now in progress.

The present work revealed that the slow component of total tritium retention curve can be interpreted by low turnover rates of lipid-,DNA- and protein-bound tritium. The largest contribution to the total tritium activity in the spleen,the liver and the lung was found for the lipid component (44%), the DNA (51%) and the protein component (37%), respectively. Thus for each

organ there were molecular components critical for dose accumulation.

In our experiments, tritium was given to suckling mice with organs still in growth. This may have given differences of tritium metabolism from adult, resulting in the difference of the critical molecular components as well. Among the organically bound tritium components, DNA-bound tritium exhibited a high steadiness against total activity per organ in a long-term experiment compared with other components. For instance, in the brain and liver, essentially no decrease was found in DNA-bound tritium activity for the period between 7 and 31 weeks after birth. It is noteworthy that such high steadiness of the content of DNA-bound tritium in organ was found also for the intestine; the tritium activity per whole organ at 7 weeks was 69 ± 21 Bq/organ at at 31 weeks, 49 ± 18 Bq/organ. The biological half-life of the slow component of tritium retention for intestinal DNA is then roughly estimated at 332 days.

The biphasic tritium retention curve for DNA-bound tritium was reported also for pregnant mice given ^{3}H-thymidine (16) ; a sizable part of the DNA, particularly in neonatal mice persited with a slow or no turnover . From an experiment using mice administered with tritiated water during the period between 4 and 8 weeks after birth, other authors reported that the biological half-life of DNA-bound tritium in the brain was 593 days with 95 % confidence limits of 376 to 1406 days (17). These findings and the present results as well indicate that DNA-bound tritium is highly stable compared with other organically bound tritium. At present, it is unclear whether the highly stable DNA-bound tritium exists only in newborn or young animals.

Two explanations are possible for the biphasic tritium retention curve of DNA-bound tritium pèr organ. One is the existence of cell populations with different tritium retention rates. The other is the lower efficiency of reutilization of DNA in cell renewal in the initial stage of animal growth compared to the later one. Further experiments are needed to solve the

problem.

The high steadiness of DNA-bound tritium in organs is important in the estimation of committed absorbed dose due to tritium intake in young mammals including human juveniles. Table 3 shows the absorbed dose accumulated for the period between removal of the tritium source and 31 weeks after birth. The committed dose equivalent (H_x) in a particular organ can be defined in analogy with the human case as the total dose equivalent to which that organ would be committed during the x weeks after intake of tritium (15). When tritium was given to suckling mice, the contribution of DNA-bound tritium to the committed dose equivalent would become greater if the period of dose accumulation is extended to the life span of the animals.

With regards to practical radiation protection a modifying factor should be considered for microdistribution of tritium in organs or tissues(10). Our results gave the organ-specific values of about between 2 and 6 as the dose modifying factor for DNA-bound tritium in newborn mice. The modifying factors could be applied for the case of a long-term exposure to tritium to determine the committed dose equivalent if essentially no differences exist in the nuclear size and DNA content per cell nucleus between young and aged mice for various organs. By using the values of biological half-life and tritium content of each molecular component, the contribution of each component to the total committed dose equivalent could be evaluated. Such data as well as the information on the microdistribution of tritium in the cell will serve for evaluating possible biological effects of tritium intake by animals including man.

References

1. L. E. Feinendegen, E. P. Cronkite and V. P. Bond, Radiation problems in fusion energy production. Radiat. Environm. Biophys. 18, 157-183 (1980).

2. D. F. Cahill and C. L. Yuile, Tritium : Some effects of continuous exposure in utero on mammalian development. Radiat. Res. 44, 727-737 (1970).

3. S. Zamenhof and E. van Marthens, The effects of chronic ingestion of tritiated water on prenatal brain development. Radiat. Res. 77, 117-127 (1979).

4. S. Zamenhof and E. van Marthens, The effects of pre- and postnatal exposure to tritiated water for five generations on postnatal brain development. Radiat. Res. 85, 292-301 (1981).

5. C. Streffer, D. van Beuningen and S. Elias, Comparative effects of tritiated water and thymidine on the preimplanted mouse embryo in vitro. In Current Topics in Radiation Research Quarterly 12, 182-193 (1977).

6. J. W. Laskey and S. J. Bursian, Some effects of chronic tritium exposure during selected ages in the rat. Radiat. Res. 67, 314-323 (1976).

7. R.J. Haas and T. M. Fliedner, The effect of tritiated thymidine on the oocytes of foetal rats following maternal infusion in pregnancy. Int. J. Radiat. Biol. 19, 197-200 (1971).

8. H. Takeda, Tritium metabolism in rat tissues, In Proceedings of the Workshop on Tritium Radiobiology and Health Physics (H. Matsudaida, T. Yamaguchi, T. Nakazawa and C. Saito, eds.) pp. 187-204. NIRS-M-41, National Institute of Radiological Sciences, Chiba (1982).

9. M. Saito, C. Streffer and M. Molls, Tritium distribution in newborn mice after providing mother mice with drinking water containing tritiated thymidine. Radiat. Res. 95, 273-297 (1983).

10. ICRP publication 30, Limits for Intakes of Radionuclides by Workers, Part I, Annals of the ICRP, Vol.2 No3/4, Pergamon Press, Oxford (1979).

11. A. A. Moghissi, M. W. Carter and E. W. Bretthauer, Further studies on the long term evaluation of the biological half-life of tritium. Health Phys. 23, 805-806 (1972).

12. W. C. Russel, C. Newman and D. H. Williamson, A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253, 461 (1975).

13. M. Saito, M. R. Ishida, C. Streffer and M. Molls, Estimation of absorbed dose in cell nuclei due to DNA-bound tritium. Health Phys. (1984) in press.

14. K. Tägder and W. Scheuermann, Estimation of absorbed doses in the cell nucleus after incorporation of ^{3}H- or ^{14}C-labelled thymidine. Radiat. Res. 41, 202-216 (1970).

15. ICRP publication No.26, Recommendations of the International Commission on Radiological Protection, Annals of the ICRP, Vol.1 No.3, Pergamon Press, Oxford (1977).

16. G. B. Gerber and J. Maes, Incorporation and turnover of tritium in neonatal mice and their mothers after feeding tritiated thymidine during pregnancy. Health Phys. 40, 755-759 (1981).

17. A. L. Carlsten, A. Brooks, S. L. Commerford and E. P. Cronkite, Genetic and somatic effects in animals maintained on tritiated water, In Proceedings of the Workshop on Tritium Radiobiology and Health Physics (H. Matsudaira, T. Yamaguchi, T. Nakazawa and C. Saito, eds.) pp. 101-109. NIRS-M-41, National Institute of Radiological Sciences, Chiba (1982).

NIRS-M-52, pp 35 - 49 1985

Studies of the Tritium Uptake in Some Edible Plants and Transfer to the Rat

K.Arai, H.Takeda and T.Iwakura

Division of Environmental Health
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

In our laboratory, attention has been focused on the study of tritium metabolism in plants, in particular the incorporation of tritium into some important edible plants.

The soybean plants were given tritiated water by a single irrigation in the plant culture chamber. Samples of plant tissues were collected and combusted in a Packard Sample Oxidizer and their radioactivity was subsequently determined. The highest incorporation of tritium was found in the bean, followed by the root. This distribution was quite similar to that found in other species of plant.

Other experiments demonstrated the importance of the time of administration of the tritium and the time of harvest on the distribution of tritium in the leaf, pod

and seed. Similar findings were also obtained in rice and wheat.

Rats were given a single ingestion of tritiated wheat and rice. Tritium distribution in the tissues was examined and compared to that following administration of tritiated water. Tissue retention was higher after administration of tritiated food than after tritiated water. Differences in tritium activity among the various tissues were considerable. One major reason for this is the difference in the distribution of organically bound tritium among the tissues examined.

INTRODUCTION

Tritium released from nuclear facilities into the environment is generally converted to tritiated water(HTO). In this form, tritium enters readily into living matter including important edible plants. Tritiated water is partly incorporated into organic molecules by metabolic (1,2) or photosynthetic processes (3,4,5). Therefore, human exposure to tritium may occur not only from HTO but also from tritiated organic compounds taken as food.

Some investigators (6 - 10) have reported that ingestion of tritiated food leads to a higher tritium retention in the tissue constituents of animals than that following HTO administration. Kirchmann et al (8,11), moreover, observed that the rate of tritium incorporation depends on the kind of tritiated food administered.

In the present study, edible plants including rice and wheat were given tritiated water by a single irrigation in the plant culture chamber. The distribution of tritium among the tissues of plant was subsequently determined. The results showed a definite but complex pattern of tritium distribution in accordance with the period of growth during which the tritium was given and the time of the harvest of the plants.

Then, tritiated wheat and rice were administered to the rat by a single ingestion and tissue retention of tritium was examined in comparison with that following HTO.

MATERIALS AND METHODS

Plant Experiments

The water weed, *Ceratopteris pterioides* was grown in water containing HTO at a concentration of 8 mCi/l under conditions of natural lighting for 3 and 28 days at room temperature.

Soybean, *Glycine max* L. Merr. cv. Hakucho, wheat, *Triticum vulgare* Vill. cv. Norin 26, and rice, *Oriza sativa* L. cv. Koshihikari were grown in a 1/50 m^2 plastic Wagner's pot. They were irrigated with water and nutrient solution consisting of Peters Fertilizer (W.R Grace & Co.). A metabolic chamber was used for administration of tritium to the edible plants. The chamber was closed tightly and tritiated water was usually given in a single irrigation at indicated doses. The plants were cultivated under

natural conditions of light, temperature and humidity.

The plant samples were collected at various periods of culture and separated into water and dried materials with a freezing technique. Dried samples were combusted in an oxidizer (Model 306 Tri-Carb Packard Instrument Co.) and the radioactivity in the combustion water was determined subsequently in a Beckman LS-7500 liquid scintillation counter. Results were expressed in Ci per g of tissue water or dried material.

Animal Experiments

Male Wistar strain rats aged 4-5 months and weighing about 390 g were used. The wheat and rice were grown under the conditions described above and harvested in ripening season and freeze-dried. The HTO (6.0 μCi/g body weight), tritiated wheat (2.0 nCi/g body weight) and rice (4.0 nCi/g body weight) were given to the rat in a single ingestion using stomach tube. After the ingestion, the animals were killed at 24 hrs. and various tissue samples were obtained.

A part of each tissue was weighed immediately and total tritium activity in wet tissue was determined. Another part was lyophilized with liquid nitrogen for analysis of non-volatile tritium activity in dry tissue. Lyophilization was repeated twice by adding distilled water to remove water-form tritium. Tritium activity was determined as described above.

Volatile tritium activity was calculated by substracting non-volatile tritium activity from total tritium activity

of the same sample. The volatile tritium, therefore, refers to tritium in tissue water, namely tritiated water. All data were expressed as relative concentration which was calculated as follow :

$$\text{relative concentration} = \frac{\text{activity per g sample weight}}{\text{activity administered per g body weight}} \times 100$$

Relative concentration of volatile tritium for each tissue was calculated using the water content of each tissue which had been reported in our previous publication (12-13).

RESULTS

Tritium metabolism in the plant

Fig. 1. shows typical changes in the distribution of tritium during a continuous administration of tritiated water to water weed. Tritiated water became equilibrated very quickly with free water of the tissue reaching a plateau by 1 hour, while that incorporated into dried matter increased slowly.

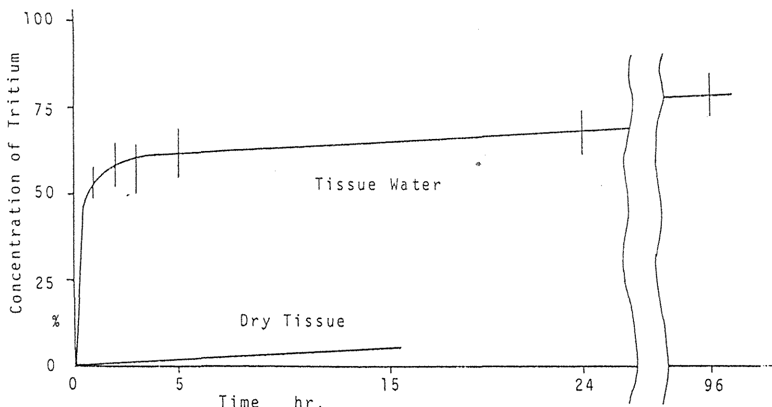


FIG. 1. Distribution of tritium activity in *Ceratopteris pterioides*

Table 1. shows tritium concentration in free water and in dried matter, that is, in the non-volatile fraction of soybean leaves. Tritiated water was given as a single administration at 48 days after sowing. Leaf samples were collected after 24 hours. The concentration in free water is nearly constant among different leaves taken from the bottom to the top of the plant. The concentration in the dried matter is from about 2 to 11 percent of that found in free water and varied according to the position of leaves, higher in the upper leaves.

TABLE 1.

TRITIUM ACTIVITY in SOYBEAN LEAVES (pCi/mg)

Leaf No.	Tritium Activity in Free Water	Tritium Activity in Dried Tissue
2	1310	21.4
3	1240	44.1
4	1320	93.6
5	1250	134
6	1260	132

administration, 20 mCi/pot, 48 days after sowing. sampling, 24 hours later.

TABLE 2.

TRITIUM ACTIVITY in DRIED TISSUES of SOYBEAN (pCi/mg)

Tissues	Tritium Activity
Root	1500
Stem	900
Leaf	600
Pod	700
Seed	1900

administration, 250 mCi/pot, 85 days after sowing. sampling, about 2 weeks later.

Table 2. shows the tritium concentration, in this case non-volatile tritium, in dried tissues of soybean. Tritiated water was administered in a single irrigation at 85 days after sowing and tissue samples were collected at the natural harvest time, that is about 2 weeks after administration. Tritium activity is highest in the seed, followed by the root. The lowest was found in the leaf.

Table 3. shows influences of time of administration and stage of growth of soybean plants on the concentration

of non-volatile tritium in soybean. Tritium concentrations in the pod and seed varied considerably with time of the administration. Administration at the biginning of August, that is the time of ripening of soybean, followed by harvest 2 or 3 weeks later, was the most effective means in obtaining soybean seeds of high radioactivity.

TABLE 3. UPTAKE of TRITIUM in SOYBEAN DRIED TISSUES (pCi/mg)

No.	POD	SEED
1-4	31.4	9.86
1-5	29.1	7.73
2-1	10.9	10.5
2-2	12.3	65.9
2-3	9.32	60.5
3-1	2.50	1.82
3-2	6.36	12.7

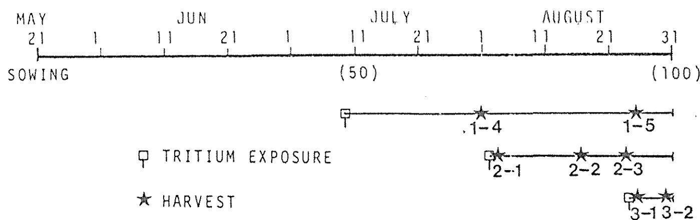


Table 4. shows changes in the uptake and distribution of non-volatile tritium in wheat grains as a function of time after flowering and administration. Tritium concentration in wheat fruit was highest when tritiated water was administered at two weeks after flowering, the period where growth of the fruit is very rapid.

Table 5. shows the distribution of non-volatile tritium, in different tissues of the rice. Tritiated water was given at about two weeks after flowering and samples were collected about 50 days later, at the natural time of

harvest. The fruit portion of the rice grain contained the highest radioactivity which was about ten times that found in stems and leaves.

The samples of wheat fruit and rice tritiated under the optimum conditions given in Tables 4 and 5 were used in the animal experiments which will be described below.

TABLE 4.

DISTRIBUTION of TRITIUM ACTIVITY in WHEAT GRAINS (pCi/mg)

Days	Fruit	Shell
1	250	932
15	912	73
25	195	13
37	99	10

HTO 10 mCi/300 ml/pot

TABLE 5.

DISTRIBUTION of TRITIUM in *Oryza sativa L.* (pCi/mg)

Tissues	Tritium Activity
Rice	3760
Shell	710
Stem	340
Leaf	390

500 mCi/500 ml/pot administered at 2 weeks after flowering and samples harvested 50 days later.

Tritium metabolism in the rat

Table 6. summarizes data of tritium distribution in rat tissues at 24 hours after a single ingestion of HTO, tritiated leucine and tritiated glucose. The results were expressed in relative concentration (see, MATERIALS & METHODS). Considerable differences can be seen in the values of non-volatile tritium between tritiated water and tritiated leucine. The results of tritiated glucose were intermediate of these two. The values for volatile tritium did not vary much among the tissues examined except for fat tissue nor between the compounds administered.

Table 7. gives similar data at 24 hours after a single ingestion of HTO, tritiated wheat and tritiated rice. The

TABLE 6.

DISTRIBUTION of TRITIUM in RAT TISSUES
at 24 HOURS AFTER INGESTION of TRITIATED COMPOUNDS

Tissue	Relative concentration					
	Tritiated water		Tritiated leucine		Tritiated glucose	
	Volatile	Non-volatile	Volatile	Non-volatile	Volatile	Non-volatile
Liver	104	10	59	297	92	40
Kidney	101	6.7	62	381	104	28
Testis	92	5.5	42	187	108	28
Spleen	99	7.3	45	223	104	34
Brain	97	4.8	52	96	91	37
S. intestine	101	8.2	--	--	--	--
Fat tissue	23	0.3	15	5.3	31	8.6

$$\text{Relative concentration} = \frac{\text{radioactivity per g sample weight}}{\text{radioactivity administered per g body weight}} \times 100$$

data for HTO were taken from Table 6. The concentration of volatile tritium found among the tissues examined after tritiated wheat and rice ingestion were not significantly different from each other, being at levels similar to those

TABLE 7.
DISTRIBUTION of TRITIUM in RAT TISSUES
at 24 HOURS AFTER INGESTION of TRITIATED WATER and FOODS

Tissue	Relative concentration					
	Tritiated water		Tritiated wheat		Tritiated rice	
	Volatile	Non-volatile	Volatile	Non-volatile	Volatile	Non-volatile
Liver	104	10	80	310	106	129
Kidney	101	6.7	74	204	99	107
Testis	92	5.5	69	173	108	69
Spleen	99	7.3	69	212	108	123
Brain	97	4.8	84	108	113	64
S. intestine	101	8.2	65	257	83	246
Muscle	103	4.3	85	100	115	43
Fat tissue	23	0.3	13	26	73	40

$$RC = \frac{\text{radioactivity s.w./g}}{\text{radioactivity administered b.w./g}} \times 100$$

found after HTO ingestion. On the other hand, the concentrations of tritium in dry tissues, i.e., non-volatile tritium, were appreciably different among the tissues examined. Moreover, the concentrations were higher by a factor of 10-30 than those found after tritiated water ingestion.

DISCUSSION

When tritiated water is given to a plant, it is taken up into the tissues and distributed homogenously as free water. A portion is incorporated into the organic matter via metabolism and constitutes organically bound tritium of the tissue.

As shown in Fig. 1, the concentration of tritium in free water of water weed remained almost constant until the end of continuous administration. On the contrary, the concentration of tritium in dry matter increased very slowly during the experimental period. The maximum tritium concentration in tissue water never attained the tritium concentration level of administration water. The reason for this is not well understood. Similar results were reported by Shibabe who examined the uptake of D_2O in rice plant (14).

By comparing the results of tritium incorporation into several tissues of soybean after a single administration of tritiated water, it is possible to assess accurately the differences in tritium incorporation into different tissues of the plant. The highest incorporation of tritium

occurred in the bean, followed by the root. The results were quite similar to those reported by Cline (15).

The tritium concentration in free water in the leaves of soybean of 24 hours after administration was found to be fairly constant irrespective of their position, whereas, the concentration in dried tissue was higher in upper leaves.

The tritium concentration in leaf, pod and seed of soybean showed a complex but definite pattern reflecting the times of administration and harvest. This may reflect the differences of metabolism in the tissues during the life cycle of the plant. The same may apply to the case of rice and wheat. Considerable differences of tritium incorporation were found, depending on the times of administration, between the fruit and shell of wheat grains, and rice and shell of rice grains.

Tritium concentration in wheat fruit was highest when tritiated water was administered at 2 weeks after flowering, the period where growth of the fruit is very rapid.

A concentration of about 4000 pCi/mg was obtained after administration of a large amount of tritiated water, that is 500 mCi/500 ml/pot. Fruits of rice grain with this concentration of tritium, however, when administered to the rat by a single ingestion enabled us to study the tissue retention only for the initial five days. Larger amounts of tritiated water are necessary to prepare rice with a higher concentration of tritium, to be able to

follow the tissue retention for longer periods of time.

Several investigators have performed comparative study of tritium metabolism after chronic ingestion of tritiated water and some tritiated foods. Pietrzak-Flis (7) showed that the incorporation of tritium into organic components of tissues is higher in rabbits given tritiated alfalfa than those given tritiated water. The greatest difference was found in the bone marrow where the incorporation from tritiated alfalfa was as much as 9 times higher than that from tritiated water.

Also, Rochalska (6) showed that tritium concentrations in dry tissues of rats given organically-bound tritium were significantly higher than those given tritiated water. The ratio of tritium concentration in dried tissues after tritiated meat and tritiated water ranged from 17 in the small intestine to 3 in the brain.

The concentrations of non-volatile tritium in rat tissues differed from each other depending on the types of tritiated foods or compounds.

Tritium incorporation into organic constituents of tissues of rats after a single ingestion of tritiated leucine and tritiated glucose was higher than after ingestion of tritiated water. The absorbed dose was calculated for unit intake of each compound for various tissues. Doses after tritiated leucine were about three times higher than those after tritiated water. The contribution of the non-volatile fraction to the total dose was

about eighty percent after tritiated leucine, whereas that after tritiated water was less than ten percent.

The tritium incorporation into organic constituents of tissues of rats at 24 hours after a single ingestion of tritiated wheat and rice was as much as 10-30 times higher than after ingestion of tritiated water. Similar results were obtained at 3 and 5 days after the administration. Results with tritiated wheat resembled more those with tritiated leucine, whereas those with tritiated rice, more closely resembled those with tritiated glucose.

CONCLUSION

The incorporation of tritium into animal tissue, particularly into organic molecules, was found to be considerably high when it was given as tritiated foods. The uptake of tritiated water into the edible portion of certain plants was, however, confined to a specific period of the growth, indicating the importance of the time of tritium release into the environment in the assessment of human exporure.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Matsudaira for his encouragement and valuable suggestions. This work was supported by a special grant for Tritium Research from the Science and Technology Agency and in part by the Grant-in-Aid for Fusion Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. T.E.Smith and R.T.Taylor, Incorporation of tritium from tritiated water into carbohydrate, lipid and nucleic acids. Report No. UCRL-50781. (1969).

2. F.T.Hatch and J.A.Mazrimas, Tritiation of animals from tritiated water. Radiat. Res. 50, 339-357 (1972).

3. I.C.Choi and S.Aronoff, Photosynthate transport using tritiated water. Plant Physiol. 41, 1119-1129 (1966).

4. T.Kanazawa, K.Kanazawa and J.A.Bassham, Tritium incorporation in the metabolism of chlorella pyrenoidosa. Environ. Sci. Technol. 6, 638-642 (1972).

5. S.Strack, G.Kistner, C.C.Emeis, Incorporation of tritium into planctionic algae in a continuous culture under dynamic conditions. In:Proc. Symp. Behaviour of tritium in the envirionment. 219-230. San Francisco, CA. IAEA SM-232/40 (1979).

6. M.Rochalska and Z.Szot, The incorporation of organically-bound tritium of food into some organs of the rat. Int. J. Radiat Biol. 31, 391-395 (1977).

7. Z.Pietrzak-Flis, I.Radwan and L.Indeka, Tritium in rabbits after ingestion of freeze-dried tritiated food and tritiated water. Radiat. Res. 76, 420-428 (1978).

8. R.Kirchmann, P.Charles, R.Van Bruwaene, J.Remy, G.Koch and J.Van den Hoek, Distribution of tritium in the differint organs of calves and pigs after ingestion of various tritiated feeds. Curr. Top. Rad. Res. Quart. 12, 291-321 (1977).

9. J.Van den Hoek and M.H.J.ten Have and G.B.Gerber, The metabolism of tritium and water in the lactating dairy cow. Health Physics 44, 127-133 (1983).

10. Z.Pietrzak-Flis, I.Radwan, Z.Major and M.Kowalska, Tritium incorporation in rats chronically exposed to tritiated food or tritiated water for three successive generations. J. Radiat. Res., 22, 434-442 (1981)

11. R.Van Bruwaene, G.B.Gerber, R.Kirchmann, J.Van den Hoek and J.Vankerkom, Tritium metabolism in young pigs after exposure of the mothers to tritium oxide during pregnancy. Radiat. Res., 91, 124-134 (1982).

12. H.Takeda and Y.Kasida, Biological behavior of tritium after administration of tritiated water in the rat. J. Radiat. Res., 20, 174-185 (1979).

13. H.Takeda, Comparative metabolism of tritium in rat after a single ingestion of some tritiated organic compounds versus tritiated water. J. Radiat. Res., 23, 345-357 (1982)

14. S.Shibabe and K.Yoda, Hydrogen isotope effect on transport of potassium ion in rice seedlings equilibrated with deuterium oxide. Radioisotopes, 33, 675-679 (1984).

15. J.F.Cline, Absorption and metabolism of tritium oxide and tritium gas by bean plants. Plant physiol., 28, 717-723 (1953).

NIRS-M-52, pp 50 - 61 , 1985

Metabolism of Organically Bound Tritium

C.C.Travis

Health and Safety Research Division
Oak Ridge National Laboratory
P.O.Box X, Oak Ridge, Tennessee 37831, U.S.A.

Abstract

The classic methodology for estimating dose to man from environmental tritium ignores the fact that organically bound tritium in foodstuffs may be directly assimilated in the bound compartment of tissues without previous oxidation. We propose a four-compartment model consisting of a free body water compartment, two organic compartments, and a small, rapidly metabolizing compartment. The utility of this model lies in the ability to input organically bound tritium in foodstuffs directly into the organic compartments of the model. We found that organically bound tritium in foodstuffs can increase cumulative total body dose by a factor of 1.7-4.5 times the free body water dose alone, depending on the bound-to-loose ratio of tritium in the diet. Model predictions are compared with empirical measurements of tritium in human urine and tissue samples, and appear to be in close agreement.

Operated by Martin Marietta Energy Systems, Inc. with the U.S. Department of Energy under Contract DE-AC05-840R21400

INTRODUCTION

Tritium, the heaviest and only radioactive isotope of hydrogen, is produced naturally as a result of cosmic ray interactions in the stratosphere. Significant quantities of tritium are also released to the environment through nuclear weapons testings, nuclear power reactors, fuel reprocessing plants, and consumer products. Once released to the atmosphere, tritium is oxidized to tritiated water (^{3}HOH), enters the global hydrological cycle, and is widely dispersed throughout the environment. Transfer to man is by inhalation, skin absorption, or ingestion of food or drinking water. Once in the human body, tritium mixes rapidly with extracellular and intracellular tissue water, with a fraction replacing hydrogen bound in organic molecules of tissue. Loose tritium is defined as that which can be removed from plant or animal tissue by mild distillation techniques or lyophilization. Bound tritium is that which remains after this process and which may be removed by combustion. Bound tritium is composed of both stable tritium (that which is primarily attached to carbon and is usually only released during enzyme-mediated reactions) and labile tritium (that which is attached to oxygen, sulfur, nitrogen or phosphorus and will readily exchange with tritium in tissue water).

Various models have been proposed to describe the kinetics of tritium in the human body. The most widely accepted of these is Bennett's three-compartment model (1) which has been adapted by the National Council on Radiation Protection (NCRP) (2). The NCRP model is presented in Figure 1. This model assumes that all ingested tritium, whether organically bound or free, enters directly into the free body water compartment. On the basis of predictions made by this model, the NCRP (2) indicates that organically bound tritium in the body may be adequately accounted for by multiplying the free body water dose by a factor of 1.2.

All tritium models currently in use ignore the fact that organically bound tritium in foodstuffs may be directly assimilated in the bound compartments of body tissue without previous oxidation. We have developed a fourcompartment

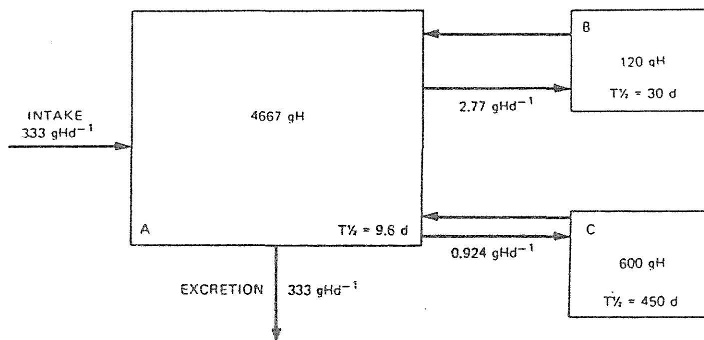


Figure 1. National Council of Radiation Protection and Measurements Three-Compartment Model of Hydrogen in the Body.

model of hydrogen metabolism in the body (see Figure 2) which allows for input of organically bound tritium directly into organic compartments representing tissue solids (3). Predictions made with this model indicate that properly accounting for metabolism of organically bound tritium in foodstuffs can increase cumulative dose estimates by as much as a factor of four to five over doses estimated for free body water alone.

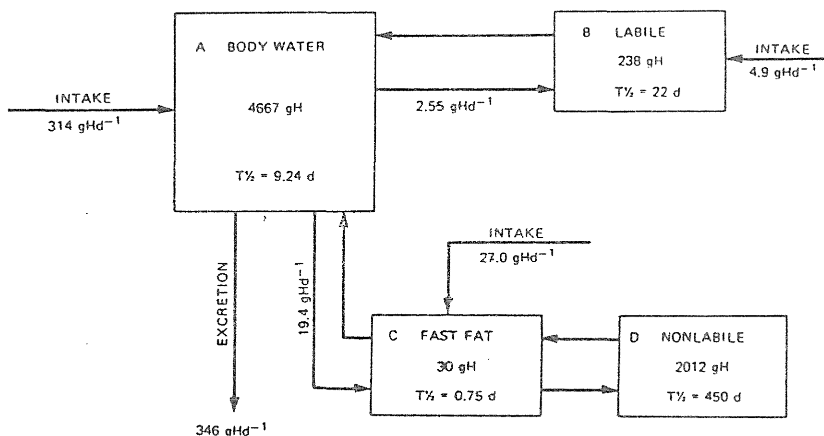


Figure 2. Four-Compartment Model of Hydrogen Metabolism.

While our model structure is similar to previous models (we have four compartments compared to two or three), the primary difference lies in the ability to input organically bound tritium directly into the compartments representing tissue solids, rather than into the free body water compartment. Model parameters

(transfer rates and compartment masses) were selected so that the response to a pulse of tritiated water input directly into the water compartment (compartment A) would duplicate the tritium retention data reported by Snyder et al. (4) and Sanders and Reinig (5) (see Figure 3). The purpose of this paper is to discuss the dosimetric implications of our four-compartment model and to present a preliminary validation using measurements of background levels of loose and bound tritium in Italian subjects and their diets.

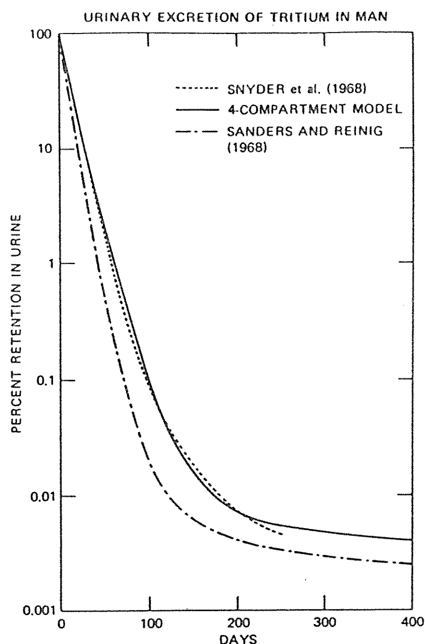


Figure 3. Urinary excretion of Tritium in Man. The two Doted Lines are empirical tritium retention data and the solid line is the 4-compartment model retention curve.

DOSIMETRY

Our primary purpose in developing a compartmental model of hydrogen metabolism was to obtain better indications of the dosimetric consequences of human exposure to organically bound tritium. To answer this question, we input a single tritium pulse of 1 μCi/kg into both Bennett's model and our four-compartment model. In Bennett's model, the entire intake was input into the body water compartment, whereas in the four-compartment model, the intake was divided proportionately into compartments A, B, and C, based on the fraction of daily hydrogen intake values (A=0.908; B=0.014; and C=0.078). Using Bennett's

model, cumulative total body dose in μCi days/kg after 2000 days is 20.33. As should be the case, this dose is approximately 1.2 times the dose from the free body water compartment alone (16.49 μCi days/kg). Our four-compartment model, which allows for input of organically bound tritium directly into the compartments representing tissue solids, yields a cumulative dose after 2000 days of 29.5 μCi days/kg, a factor of 1.7 times the dose contributed by the free body water compartment.

In estimating body burdens, the ratio (R) of total bound to total loose tritium (including drinking water) ingested daily by an individual is an important quantity. Under conditions where tritium is uniformly dispersed in the environment (equilibrium conditions), tritium has the same specific activity in both the bound and loose compartments (i.e., the bound to loose ratio which is defined as the ratio of the specific activity in the bound compartment to that in the loose compartment, is 1). However, under equilibrium conditions the R value for total ingested tritium is low (R=0.15) since the average human diet is composed of much more loose than bound hydrogen (and consequently tritium). In order to determine the effect of the parameter R on dose estimations, we repeated our dose calculation again using a single pulse input of 1 μCi/kg and varying the bound-to-loose ratio in foodstuffs. Figure 4 shows the ratio of cumulative total body dose to free body water dose after 2000 days (as predicted by the four-compartment model) as a function of the ratio R. Bennett's model estimates are included for comparison. The figure shows that for a hypothetical diet in which the total intake of bound tritium is ten times larger than the total intake of loose tritium (R=10), cumulative total body dose after 2000 days would be a factor of 4.5 times higher than the dose contributed by the free body water along.

In summary, under conditions of a unit bound-to-loose tritium ratio in individual foods items, Bennett's model predicts a cumulative dose to the body after 2000 days that is 1.2 times the free body water dose, whereas our model predicts a cumulative dose 1.7 times the body water dose. Under the dietary

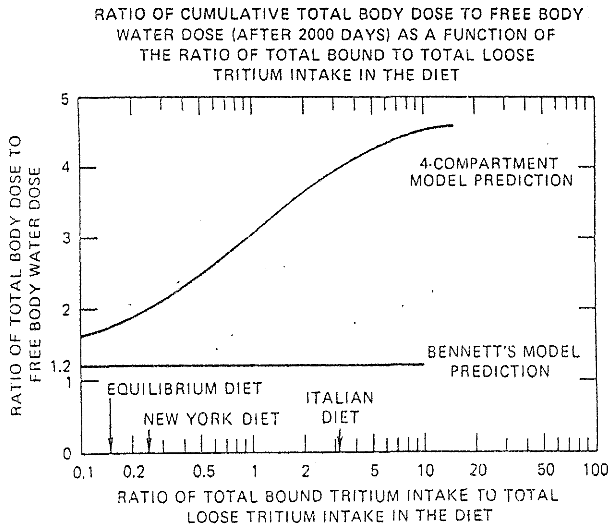


Figure 4. Ratio of Cumulative Total Body Dose to Free Body Water Dose (after 2000 days) as a Function of the Ratio R of Total Bound to Loose Tritium Intake in the Diet.

conditions described by Bogen (6), the four-compartment model predicts a cumulative dose two times that predicted for the free body water compartment. For higher bound-to-loose ratios in the diet (up to R=10), the cumulative dose could be up to 4.5 times that of the free body water compartment, while Bennett's model still predicts a ratio of 1.2. The difference here lies in the capability to input organically bound tritium directly into the organic compartments of the four-compartment model.

VALIDATION OF FREE BODY WATER COMPARTMENT

Belloni et al.[1] summarized the findings of a study on background tritium content in the Italian diet and its transfer to man. Daily dietary intake and

1. Belloni, P., Clemente, G. F., Di Pietro, S., and Quaggin, S. (1979). Data Preliminari Sullo Studio del Trasferimento di Bassi Livelli di Tritio Dall' Ambiente All' Uomo, Comitato Nazional Energia Nucleare Technical Report, RT/PROT(79)5, Rome.
2. Belloni, P., Clemente, G. F., Di Pietro, S., Quaggia, S., and Santaroni, G. P. (1981). Livelli di Contaminazione da Tritio in Diete ed Alimente Italiani, Comitato Nazional Energia Nucleare Technical Report, RT/PROT(80)35, Rome.

excretion of tritium were measured for seven healthy subjects. Bound and loose concentrations were reported for foodstuffs and blood, and loose concentrations were reported for urine for each subject. Bound to loose tritium ratios measured in individual food items of the Italian diet ranged from 2.3 for flour to 48.1 for meat.[2] These measurements are contrary to the commonly held assumption that bound-to-loose ratios in food items under background conditions should be one (2,7). Bogen et al.(8) have also measured bound to loose ratios in food items in a New York diet and found them to range from 1.2 - 5.6.

The ratio (R) of dietary tritium in the seven Italian diets was found to range from 0.5 - 8.9. Tritium concentrations in urine were found to average 3.6 times tritium concentrations in the loose fraction of the diet (with a range of 0.7 - 9.0). Again, these measurements are contrary to the commonly held belief that urine concentrations will be similar to the tritium concentration in the loose fraction of the diet. These higher concentrations of tritium in urine result from long-term retention of bound tritium in the body and its subsequent release into the body water compartment. Using data on the dietary intake of tritium for the seven subjects, we will first validate the free body water compartment of our model by comparing model predictions with reported concentration of tritium in the urine of each subject.

Daily intake of loose tritium for each of the seven subjects was based on estimated tritium concentrations in surface water (400 pCi/L), while daily intake of bound tritium was obtained by multiplying total intake of loose tritium by the measured value of R for each subject (Table 1). For example, to obtain the daily intake of loose tritium (I_L) for subject 1 (see Table 1), one multiplies the total daily intake of water (3.94 L/d) by surface water concentration (400 pCi/L). The daily intake of bound tritium (I_B) for subject 1 is obtained by multiplying I_L by R=3.4. Tritium inputs into compartments A, B, and C of the four-compartment model are $I_L + 0.3\ I_B$, and $0.11\ I_B$, and $0.59\ I_B$, respectively (3). Model predictions were used to estimate the concentration of tritium in the body water compartment of each of the seven subjects. The concentration of tritium in urine is assumed similar to that of the body water[3]. Table 2 lists the measured concentration in urine for each of the seven subjects, and

the values predicted by our four-compartment model and the NCRP model (2). It can be seen that the predictions of both models are very close to the measured values for all subjects except 4 and 5.

Table 1. Estimated parameters for Italian diet study

Subject	Weight[a] (kg)	Body water[a] compartment (L)	Urine[a] excreted (L/d)	Total water[b] intake (L/d)	R[a,c]
1	95	57	1.8	3.94	3.4
2	52	31	0.471	1.03	3.1
3	73	44	0.783	1.71	7.3
4	50	30	0.800	1.75	8.9
5	82	49	0.805	1.76	0.5
6	68	41	0.920	2.01	1.2
7	60	36	0.444	0.972	7.2

[a]from footnote 1.

[b]estimated from Reference Man (9) water balance data.

[c]R = the ratio of total bound to total loose tritium (including drinking water) ingested daily.

Table 2. Comparison of measured to predicted urine concentration for seven Italian subjects

	Urine concentration (pCi/mL)		
Subject	Measured values[a]	Four-Compartment model prediction[b]	NCRP Model prediction[c]
1	1.4	1.4	1.4
2	0.7	0.6	0.6
3	1.8	1.5	1.5
4	0.7	2.7	2.7
5	1.9	0.3	0.3
6	0.5	0.5	0.5
7	0.9	1.0	1

[a]from footnote 1.

[b]Based on computer runs using model in Ref. 3.

[c]Based on computer runs using model in Ref. 2.

VALIDATION OF ORGANIC COMPARTMENTS

Recently, Belloni et al.[4] have presented a summary of data on tritium content of the diet and human tissues from a sample of the Italian population (see Table 3). Using the daily intake of loose and bound tritium from Table 3 as input into our four-compartment model, we predict equilibrium loose and bound body burdens for the average Italian subject as 56,500 pCi and 90,900 pCi, respectively. This compares with the Belloni et al. estimate of 62,100 pCi and 93,960 pCi, respectively (see Table 3). A comparison between predicted and reported fresh tissue concentrations is given in Table 4. The close agreement of our model predictions with reported values indicates that our four-compartment model accurately represents the metabolism of tritium in the organic compartments of the human body (10).

Table 3. Average tritium concentration in diet and tissue samples of Italian population[a]

Body soft tissue weight	59,000 g
^3H urinary concentration	1,500 pCi/L
Total loose tritium daily intake (I_L)	1,472 pCi/d
Total bound tritium daily intake (I_B)	2,714 pCi/d
Average tissue concentration of loose tritium[b]	1.053 pCi/g
Average tissue concentration of bound tritium[b]	1.593 pCi/g
Loose tritium body burden (X_L)	62,100 pCi
Bound tritium body burden (X_B)	93,960 pCi

[a]from footnote 4.

[b]measured as fresh tissue.

3. Woodard, H. Q. (1970). The Biological Effects of Tritium, HASL-229, Health and Safety Laboratory, U.S. Atomic Energy Commission, New York, New York.

4. Belloni, P., Brener, F., Clemente, G. F., Di Pietro, S., and Ingrao, G. Tritium metabolism in the human body, Proceedings Seminar on the Environmental Transfer to Man of Radionuclides Released from Nuclear Installations, Brussels, Belgium, October 17-21, 1983 (in press).

The NCRP Model (2) as it now stands (inputting both loose and bound tritium into the free body water compartment) will not reproduce the concentration of tritium in the bound compartments as reported in the Italian study (see Table 4). These data support our claim that by not properly accounting for organically bound tritium in food, the NCRP methodology can underestimate cumulative dose from tritium by as much as a factor of 4-5, depending on the bound-to-loose ratio of tritium in the diet (3).

Table 4. Comparison of measured to predicted tritium concentrations in tissue

	Measured Values[a]	Four-Compartment Model Prediction[b]	NCRP Model Prediction[c]
Bound tritium	1.59 pCi/g	1.54 pCi/g	0.15 pCi/g
Urine concentration	1.50 pCi/mL	1.60 pCi/mL	1.60 pCi/mL

[a]from footnote 4.

[b]Based on computer runs using model in Ref. 3.

[c]Based on computer runs using model in Ref. 2.

CONCLUSION

Under conditions of exposure to tritiated water, organically bound tritium in the human body contributes little to cumulative dose. To account for this metabolic incorporation of loose tritium into human tissues, it is currently suggested that cumulative dose estimates be multiplied by a factor of 1.2. However, if exposure is through tritium bound in food, the cumulative dose from organically bound tritium in the body may be large, and must be considered separately. Application of the four-compartment model to tritium dosimetry predicts a cumulative dose varying from 1.7 - 4.5 times the dose predicted for the free body water compartment along (see Figure 4), depending on bound-to-loose ratios of tritium in the diet.

Given the potential importance of tritium in both fission and fusion nuclear field cycles, we stress the need for further research on uptake of organically bound tritium. This research should emphasize both human metabolism and microdosimetry of organically bound tritium.

REFERENCES

1. Bennett, B. G. Environmental tritium and the dose to man, pp. 1047-1053 in Proc. 3rd International Congress of the IRPA. Washington, D.C., U.S. Atomic Energy Commission, Technical Information Center, Oak Ridge, Tennessee. (1973).

2. National Council on Radiation Protection and Measurements (NCRP). Tritium in the Environment, NCRP Report No. 62, Washington, D.C. (1979).

3. Etnier, E. L., Travis, C. C., and Hetrick, D. M. (1984).Metabolism of organically bound tritium in man, Rad. Res. 100: 487-502.

4. Snyder, W. D., Fish, B. R., Bernard, S. R., and Ford, M. R. (1968). Urinary excretion of tritium following exposure of man to HTO -- a two exponential model, Phys. Med. Biol. 13: 547-559.

5. Sanders, S. M., Jr., and Reining, W. C. (1968). Assessment of tritium in man, in Proceedings of a Symposium for the Diagnosis and Treatment of Deposited Radionuclides (H. A. Kornberg and W. D. Norwood, eds.), Monographs on Nuclear Medicine and Biology, No. 2, pp. 534-542, Excerpta Medica, Amsterdam.

6. Bogan, D. C., Henkel, C. A., White, C. G., and Welford, G. A., (1973). Tritium intake in New York City, p. 639-646 in Tritium (A. A. Moghissi and M. W. Carter, Eds.), Messenger Graphics, Phoenix, Arizona.

7. International Atomic Energy Agency. (1979). Symposium on the Behavior of Tritium in the Environment, ed. S. Freeman, STI/PUB/498, IAEA, Vienna.

8. U.S. Nuclear Regulatory Commission, Regulatory Guide 1.109, Rev. 1. (1977). Calculation of Annual Doses to Man from Routine Releases of Reactor Effluents for the Purpose of Evaluating Compliance with 10 CFR Part50, Appendix I.

9. International Commission on Radiological Protection (ICRP) (1975). _Report of the Task Group on Reference Man_, Report No. 23, Pergamon Press, Oxford.

10. Travis, C. C., Etnier, E. L., and Hetrick, D. M., (1984). Validation of Metabolic Model for Tritium, _Rad. Res._ 100: 503-509.

NIRS-M-52, pp 62 - 63 ,1985

Microdosimetry of Tritium Beta Particles

A.Ito

Cyclotron Laboratory
Institute of Medical Science
University of Tokyo
Shirokane-dai, Minato-ku, Tokyo 108, Japan

Tritium β particles have special features of low energy and hence very short range, that makes the radio-biological effectiveness of tritium different from those of other standard radiations. Two different microdosimetric problems were studied to clarify the physical characteristics of tritium β particles.

First, the microscopic distribution of dose in the cell was calculated. The distribution patterns of the tritium were classified into (1) point, (2) insider, (3) surface, (4) outsider, and (5) uniform. The absorbed dose in cell nuclei for each case was calculated by the Monte Carlo method using simulated tritium β tracks. It was evident that the higher the concentration of tritium in the nuclei (model(1), (2) or (3)), the higher the dose. In the case of tritiated water (HTO), the concentration of HTO in cell nuclei is

uncertain. To evaluate the concentration of HTO, special soft X-ray beams having the same electron energy as tritium were devised that gave an uniform dose distribution over the whole cell.

Second, to study the LET effect of tritium β particles in low dose irradiation, a model calculation was presented. The physical probability of double strand breaks in DNA, due to the neighboring ionization events along a single track, was calculated and compared with standard radiations.

NIRS-M-52, pp 64 - 74 , 1985

Tritium Effects on DNA . Tritium Concentration Dependency of RBE in Aqueous Solution

T.Kada, Y.Sadaie and T. Inoue

Laboratories of Molecular Genetics and Mutagenesis
National Institute of Genetics
1111 Yada, Mishima-shi 411 , Japan

Summary

In evaluation of the genetic effects of β-irradiation from tritium, the simplest and one of most essential basal data may be the knowledge of the *in vitro* sensitivities of DNA. Tritiated water was added at different concentrations to 1x SSC buffer solutions containing purified DNA which was isolated from *B. subtilis* cells of 168 *thy* (wild as to arginine biosynthesis) and kept at 4°C. After different periods, the transforming activities (Arg^+) were determined. The inactivation efficiencies of β-irradiation from tritiated water increased more than 1000 times by lowering the concentration of β-ray source. Such an increase leveled off at a concentration of 0.001 μCi/ml. On the other hand, the number of apurinic sites was determined by means of AP-specific endonuclease of *B. subtilis* in colicin E1 plasmid DNA which

was exposed either to ^{3}H β-rays or ^{137}Cs γ-rays. In acute irradiation conditions, an RBE of 0.79 was found for production of the AP sites for tritium. Implications of these results were discussed.

Introduction

In order to evaluate the genetic effects of tritium, studies on the *in vitro* sensitivities of DNA are obviously essential. Among the systems in which biological effects of radiations are assessed without any interaction with proteins and other biological molecules, the transforming system of *B. subtilis* may be most sensitive. Deproteinized and purified DNA dissolved in standard SSC (x1) buffer solution is quite stable at 4°C at least for several months. This situation enabled us to study effects of tritium β-irradiation of extremely low levels. We previously carried out these experiments using tritiated glycerol[1] and later obtained similar results with tritiated water.[2-4]

Material and methods

Transforming DNA free of detectable protein and RNA was purified by the phenol method[5] from *B. subtilis* 168 *thy*. DNA (5 μg/ml) was dissolved in 1x SSC buffer (0.15 M NaCl, 0.015 M Na_3 citrate) containing tritiated water of different concentrations and kept at 4°C . After different periods, samples of 0.1 ml were taken , dialyzed against large amounts of isotope-free 1x SSC buffer to eliminate the radioactivity. The irradiated DNA was then mixed with competent culture (1 ml) of *B. subtilis* H17 (*arg trp*) and the radiation-induced inactivation of the Arg^{+} marker was studied. The detailed methods of preparation of competent cells and media

as well as procedures of transformation experiemtns were described.[6]

The absorbed dose of tritium β-rays was calculated with the total energy dissipated by the β-rays in 1 ml (gram) of water. Because the average energy of tritium β-rays is 0.0057 Mev,[7] HTO of 1 μCi/ml dissipates an energy of 1.2 erg in 1 ml of water per 1 hr. The DNA solutions used in the present experiments were considered essentially as water for calculation of the absorbed dose. We adopted 0.012 rad for 1 μCi/ml/hr.

To determine the number of AP sites per molecule in β- or γ-irradiated DNA, ^{14}C-labeled covalently closed circular molecules of colicin El were irradiated in a buffer consisting of 100 mM NaCl/10 mM KP, pH 7.0 at a concentration of 400 μg/ml. For β-ray irradiation, tritiated water was added to the DNA solution at a final concentration of 196 μCi/ml or 439 μCi/ml and incubated at 0°C for appropriate times. The absorbed dose was calculated according to the assumption as described above that 1 μCi/ml of HTO delivers 1.2 erg/g/hr, i.e., 0.012 rad/hr. Gamma-irradiation was performed with a ^{137}Cs source at a dose rate of 100 rad/min. After the irradiation, 30 ml aliquots of the sample were incubated with or without 17 units of AP endonuclease[8] for 10 min at 37°C and the total mixture was subjected to neutral sucrose gradient analysis. Radioactivities of tritium and ^{14}C were determined with a Beckman LS 9000 liquid scintillation spectrometer with the aid of toluene triton scintillation mixture. The number of disintegrations per minute was measured by a computer program installed to the machine.

Results

1) Concentration dependency of DNA inactivation by tritiated water

Results of inactivation of the transforming DNA by tritiated water are shown in Fig. 1 (a-d). In Figure 1a, we plotted residual activities of the Arg^+ marker of DNA treated with two different concentrations of tritiated water, namely 10 μCi/ml and 1 μCi/ml. To get the same dose, treatment with the 1 μCi/ml solution may require theoretically a period 10 times longer than that with the 10 μCi/ml solution. However, we see in this figure that the transforming DNA was inactivated much more efficiently in the 1 μCi/ml solution than in the 10 μCi/ml solution. Similar observations were made when the tritiated water sources were diluted as shown in Figs. 1b-d.

The doses for 37 % survival (D_{37}) obtained at different tritium concentrations are summarized in Figure 2. As shown in this figure, the efficiencies of β-irradiation from HTO increased more than 1,000 times by lowering concentration of the β-ray source. Such an increase leveled off at a concentration of 0.001 μCi/ml. Since the dose reducing the Arg^+ transforming activity to 37 % level was about 1.7 kR (data not shown) in the case of γ-rays from ^{137}Cs, 1 R of γ-rays corresponds to 84 μCi/ml x hr of tritium β-rays with an absorbed dose of 0.108 rad at a concentration of 100 μCi/ml. The same dose of γ-rays however corresponds also to 0.058 μCi/ml x hr, that is an absorbed dose of 0.000696 rad at concentration of 0.01 μCi/ml.

The above results obtained with tritiated water were very similar to the previous ones obtained with tritiated glycerol,[1] except that the shapes of inactivation curves

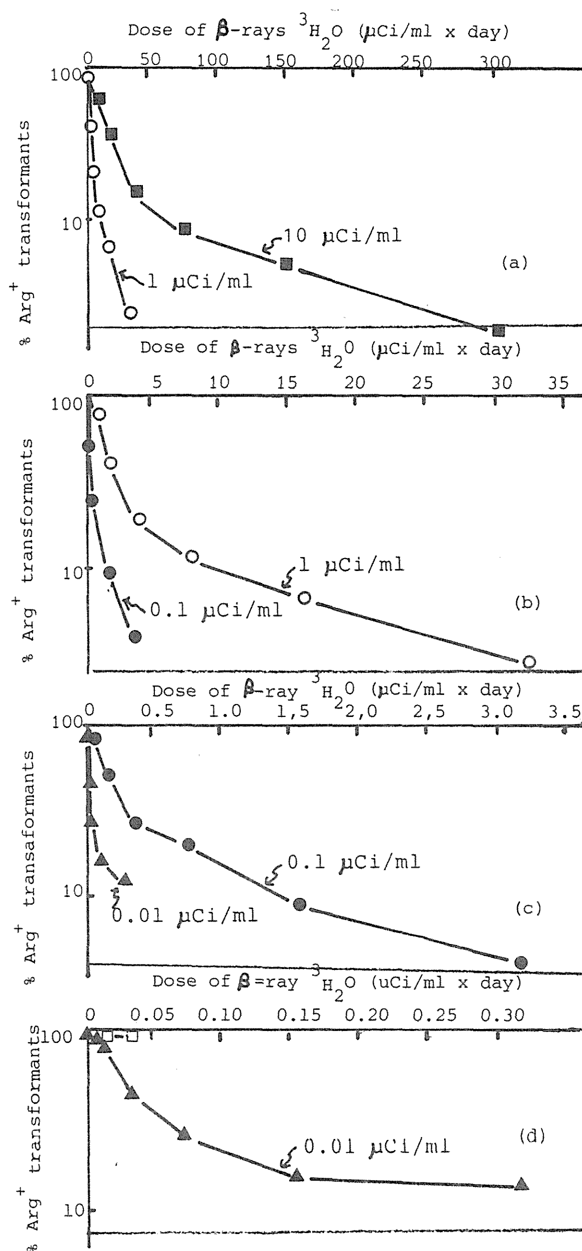


Fig. 1,a-d. Inactivation of transforming DNA of B. subtilis by beta-irradiation from tritiated water. The transforming DNA (5 µg/ml) was dissolved in 1x SSC buffer containing ^{3}H-water of different concentrations and kept at 4°C for irradiation at different dose rates (given in parentheses).

■———■ 10 µCi/ml (0.12 rad/hr)

○———○ 1 µCi/ml (0.012 rad/hr)

●———● 0.1 µCi/ml (0.0012 rad/hr)

▲———▲ 0.01 µCi/ml (0.00012 rad/hr)

□———□ 0.001 µCi/ml (0.000012 rad/hr)

See the text for details of determining the residual transforming capacity of the Arg^+ marker.

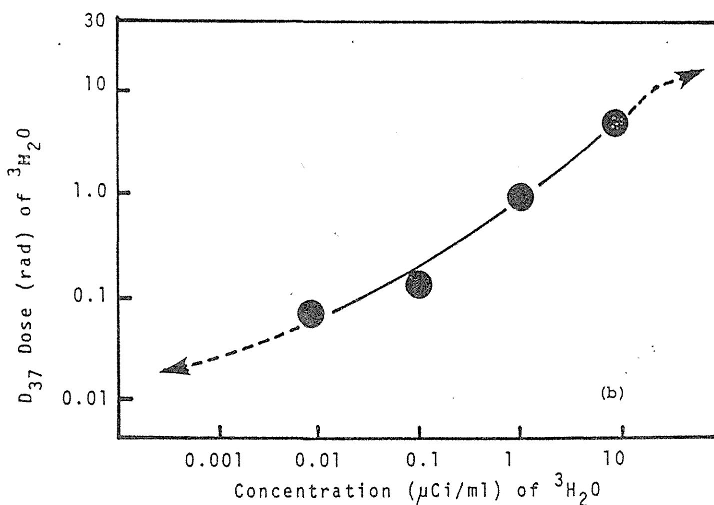


Fig. 2. Relationship between the dose of beta-irradiation giving 37 % residual Arg^+ activities of the transforming DNA and the concentration of tritiated glycerol (μCi/ml) for irradiation.

are somewhat different. In any way, the relationship between the dose of ß-irradiation giving 37 % residual Arg^+ activities of transforming DNA and the concentration of tritium was similar both for tritiated water and tritiated water.

2) Quantitative comparison of production of apurinic sites by ^{3}H-ß-irradiation and ^{137}Cs-γ-irradiation

In addition to strand scissions, ionizing radiation introduces apurinic sites (AF sites) in DNA. AP sites may have both lethal and mutagenic effects unless they are not correctly repaired in the cells. In order to quantitate biological effects of tritium we compared the number of AP sites in tritium-treated DNA with those in gamma-irradiated DNA by using purified apurinic endonuclease of *B. subtilis*.

The enzyme was purified from cell-free extract of *B. subtilis* to a near homogeneity by successive chromatographies

on DEAE-cellulose, phospho-cellulose and second DEAE-cellulose.[8] The final preparation was purified about 1000-fold and contained no activity toward DNA without AP sites. The substrate DNA, of ^{14}C-labeled colicin El DNA which has a covalently closed circular duplex structure, was made as described previously. To assess the number of AP sites, the irradiated DNA was digested with the apurinic endonculease and subjected to neutral sucrose density gradient analysis as described

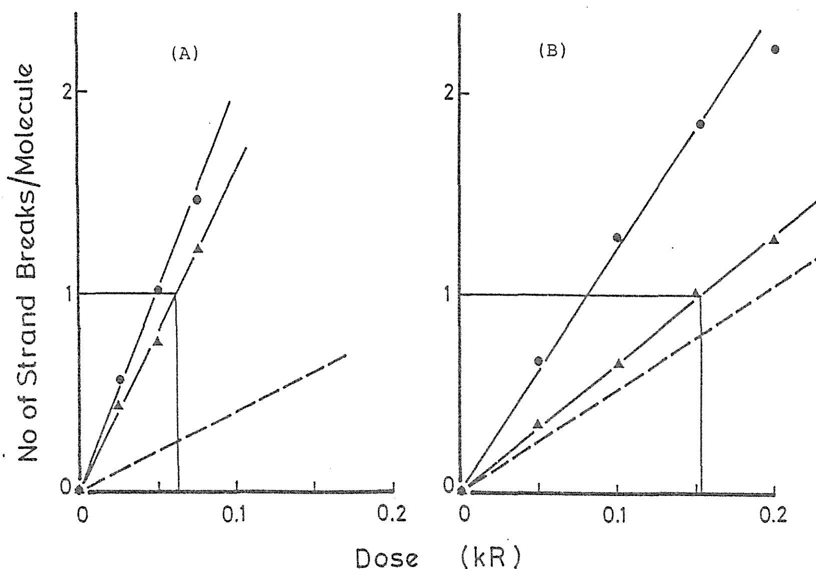


Fig. 3. Quantitative comparison of production of apurinic-sties. Details of experiments are described in the text. The number of strand breaks per DNA molecule was plotted against the absorbed doses of radiations: A; beta-irradiation from ^{3}H, and B; gamma-irradiation from ^{137}Cs.

●——● With AP endonuclease

▲——▲ Without the enzyme

— — — — Difference of the +APendo and -APendo

by Sakakibara and Tomizawa.[9] The average number of nicks per molecule was calculated from the relative radioactivity under the peaks of the covalently closed circular and open circular molecules in the sedimentation profiles.

The results are summarized in Fig. 3 where the number

of DNA strand-scissions per molecule with or without the enzyme treatment was plotted against radiation doses of beta- or gamma-rays. The differences between enzyme treated and non-treated samples were calculated and shown as dashed lines which indicate the net number of AP sites in the DNA molecules. From this figure, we can estimate that the dose of beta-irradiation producing one AP site per molecule is 0.24 kR whereas that of gamma-irradiation is 0.19 kR. Therefore, an RBE is calculated to be 0.79 for production of AP sites under these conditions.

Discussion

Since very similar results were obtained both with tritiated glycerol and water, ß-irradiation from tritium is itself considered to result in a highly efficient inactivation of transforming DNA. We checked, in certain experiments, the amount of incorporated tritium in DNA and found that it is always too small to explain the corresponding efficient activites. Therefore, we plan to examine different probable characteristics in actions of external ß-irradiation of DNA in aqueous solution. The following points are discussed:

1) Since a series of dilutions of tritiated water did not diminish the inactivating activities on transforming DNA, the simplest explanation may be that beta-radiation-produced radicals in water were "uselessly" wasted by recombination with each other. It was only at a concentration of 0.001 μCi/ml that the inactivation was most efficient, that is, more than 1000 times that of the 10 μCi/ml solution for example.

2) With every 1/10 dilution of tritiated water, we had to treat the DNA for a period longer by a factor of 10.

Therefore, we have to examine if the present observation might relate to the time factor.

(a) Perhaps beta-irradiation of tritium produces radicals of a long life or a stable reactive chemical species (including H_2O_2) then treatment with 10 unit doses for one day is equivalent to treatment with one unit dose for 10 days. This situation is schematically shown in Fig. 4. Two rectangular spaces are put equal by supposing that radicals produced at the first moment are stable in the course of treatment.

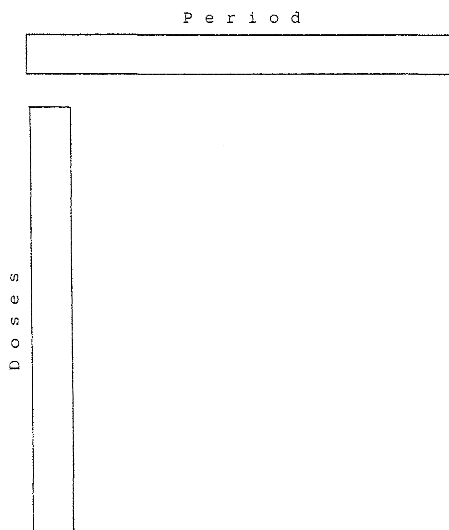


Fig. 4. Scheme of administration with stable radicals at two different concentrations for different periods.

(b) The above notion of the time factor including stability of radicals may be replaced by chain radical reactions in producing a constantly reactive chemical species in aqueous solution initiated by irradiation followed by progressive fixation of damages in DNA. In the last hypothesis, we suppose that the fixation of damage in DNA requires a long time which is a function of the period for treatment at 4°C.

We don't know at the present step what are the exact mechanisms involved. Recent preliminary experiments showed that the observed efficient inactivation of DNA depends on the concentration of buffer ingredients as well as that of the target DNA molecules. For example, at a x10 concentration of SSC, the transforming DNA is very resistant compared to the case using x1 SSC. In addition, the lesion primarily produced by tritium irradiation seems to be unstable with respect to the precipitation and washing procedure. These situations force us to imagine that the DNA lesions responsible for inactivation of the transforming capacity are strictly dependent on the conformation of DNA. For example, a lesion consisting of partial melting of DNA may be nicked by the host nuclease in the early step of transformation. This and other possibilities should be examined in the future. It is probable that such changes may be restricted to naked DNA *in vitro* but probably not to organized DNA *in vivo* in the chromosome.

Acknowledgements

The authors express their sincere thanks to Dr. Y. Tazima, former director of the National Institute of Genetics, Mishima, for his encouragement and former helpful advices on the present studies. They are also indebted to Miss M. Hara for her technical assistance. This work was supported in part by grants in aid for basic sciences from the Science and Technology Agency and from the Ministry of Education of Japan. Contribution No.1592 from the National Institute of Genetics, Mishima, Japan.

References

1) Y. Sadaie, T. Inoue, H. Mochizuki and T. Kada, Efficiencies of DNA inactivation and mutation induction by tritiated glycerol in bacterial systems. J. Radiat. Res. 22, 387-394 (1981).

2) T. Kada, H. Mochizuki, T. Inoue and Y. Sadaie, Effects of ß-irradiation from ^{3}H-water on transforming DNA of Bacillus subtilis. J. Radiat. Res. 22, 22 (1981).

3) T. Inoue and T. Kada, Enzymatic comparison of DNA damages induced by beta-rays from tritiated water and by gamma-rays. Ann. Rep. National Institute of Genetics, No. 32(1981), 91-92 (1982).

4) Y. Sadaie, T. Inoue and T. Kada, Efficiencies of DNA in-activation by tritiated water in bacterial systems. Proc. 7th Intern. Conf. Radiation Res. B1-18 (1983).

5) H. Saito and K. Miura, Preparation of transforming deoxy-ribonucleic acid by phenol treatment. Biochim. Biophys. Acta 72, 619-629 (1963).

6) Y. Sadaie and T. Kada, Recombination-deficient mutatns of Bacillus subtilis. J. Bacteriol. 125, 489-500 (1976).

7) E. A. Evans,, Tritium tracers in biology-Tritium facts or fiction? In "Tritium", pp. 659-675, Moghissi, A. A. and Corter, M. W. (ed.), Messenger Graphics, Las Vegas, Nevada.

8) T. Inoue and T. Kada, Purification and properties of a Bacillus subtilis endonuclease specific for apurinic sites in DNA. J. Biol. Chem. 253, 8559-8563 (1978).

9) Y. Sakakibara and J. Tomizawa, Replication of colicin El plasmid DNA in cell extracts. Proc. Nat. Acad. Science, 71, 802-806 (1974).

NIRS-M-52, pp 75 - 80 , 1985

Radiobiological Effects of Tritiated Water on Yeast. High Incidence of Division Anomaly of Yeast Cells in HTO Containing Growth Medium

T.Ito and A.Ito

Institute of Physics
College of Arts and Sciences
University of Tokyo
Komaba 308-1, Meguro-ku, Tokyo 153, Japan

ABSTRACT

We found division anomaly of yeast cells when incubated in HTO containing liquid growth medium over a period of several division cycles. This anomaly appeared as a beads-like structure consisting of several compartments, each being of about cell size. Each of these compartments has a nucleus but the septum to the next compartment seems to be lacking. The dose required to produce this anomaly was surprisingly small as calculated per one new compartment formation, namely, order of a few krad in terms of β-particle dose (D_{50} of non-dividing cells is about 30 krad).

INTRODUCTION

Possible effects of long-term exposure to tritium released into the environment from fusion reactor facilities is one of the recent concerns. Thus, instead of treating the cells with HTO for a period as short as possbile under non-dividing conditions (1) as usually done in most of the irradiation experiments, HTO was given in the present studies under conditions which allow cell growth during the treatment. We report here abnormalities of the cell division occurred not only at doses much less than D_{50} in non-dividing cells but also in a manner quite unique to the dividing conditions. Details will be published elsewhere.

MATERIALS AND METHODS

Cell strain and culture. A diploid *Saccharomyces cerevisiae* strain D61M, provided by Dr.F.K.Zimmermann, was used throughout. Exponentially growing cells were harvested from 13 hr YEP (Bacto yeast extract 1%,Bactopeptone 2%) medium with 2% glucose and pH 5.0 .

HTO treatment under culture. HTO was added to cell suspension in minimal synthetic medium. The cells were then incubated at 30°C in a thermoregulated box(SHD-I,Iuchi,Tokyo) for indicated periods up to 23 hr. The final concentrations of HTO used for 5 experimental series were 10.6, 14.2, 29.5, 45.5 and 98.3 mCi/ml, respectively. HTO (2 Ci ampule) was purchased from Amersham International,England. In some experiments, cells were treated with HTO in distilled water. In this case an equal amount of the cell suspension (4×10^7/ml) and HTO solution

of predetermined concentration were mixed and the mixture held at 10°C.

RESULTS AND DISCUSSION

The colony forming ability was assayed with samples taken at times during the culture designed for 16- and 20-hr treatment. Each sample had been exposed to a defined concentration of HTO for either 16 or 20 hr. The results are shown in Fig. 1. In the control, the cells were cultured under the identical conditions but in the absence of HTO. For comparison, a conventional dose-survival curve of cells held in distilled water containing HTO is also shown. As can be seen from the figure, the "survival curves" under growing conditions, though their nature is quite different from the conventional ones, were shifted considerably to the left:The cells were three times more sensitive under this culture than non-dividing condition.

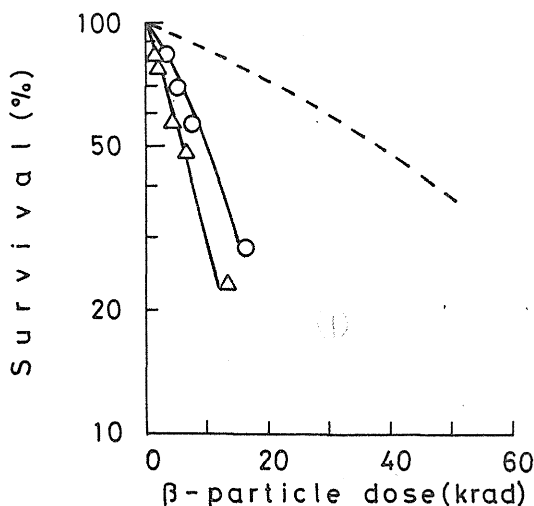


Fig. 1. "Survival curves" of yeast cells treated with HTO in growth medium. Each point was obtained after fixed culture times, 16 (Δ) and 20 (O) hr, with appropriate HTO concentrations. Dotted curve is the conventional survival curve obtained with the cells treated with HTO in distilled water.

When the samples taken at times during HTO treatment were observed under a light microscope, we found a strange change with very high frequency where several "cells" formed a linear beads-like structure. This occurred in the samples treated with HTO above 45.5 mCi/ml for 16 hr. Each compartment of the beads-like structure is constricted in a size of the cell, as if it is almost separated. The compartment was more round and larger than the normal cells. The number of the compartments in this structure was 4-6, somewhat less than the expected division number in 16 hr. It can be seen that each compartment is connected through a narrow channel as if the septum formation at the final stage of cytokinesis failed (2). If each compartment was counted as one, the total counts obtained at times during incubation were almost the same as those of the control where divided cells are singly separated.

Yeast cells have been reported to form so-called giant cells after X-irradiation with a heavy dose around 100 krad (3,4). In this case, the irradiated cells continue to grow without DNA replication nor apparent constriction. The beads-like structure characterized by several consecutive constrictions without separation is quite different from the radiation-induced giant cells. The β-ray dose for the occurrence of the beads-like structure, calculated under the assumption that 1mCi/ml of HTO is equivalent to 8.57 rad/min (1) in a water-rich system like cells, is at most 10 krad for 16 hr. Since the number of compartments in the beads-like structure had doubled every 2 to 3 hr, each compartment would receive only a fraction of 10 krad. It should be pointed out a few krad of radiation for yeast cells is equivalent to 10 rad or so for human cells.

In other series of experiments we have noticed severe

toxic effects of HTO sample from a 7 month-old ampule. They seemed to be due to some radiation products probably accumulated in the HTO sample. Unexpectedly we found that yeast cells which had been treated for 16 hr at 30 mCi/ml of HTO were severely deformed and their colony forming ability dropped to 0.01 %. The calculated β-particule dose was about 4 krad, a dose which normally did not affect the cell survival. Observations under a microscope with a Nomarsky-type interference optics (BHS-N, Olympus,Tokyo) showed that the surface of the cells was no longer smooth as in a healthy cell. Vacuoles were not visible. Later tests showed that these changes occur following treatment for less than 1.5 hr.

These morphological changes of yeast cells induced by unknown toxic product(s) are reminiscent of those in near-UV irradiated cells (5) where cell membrane damage by oxy-radicals seemed to produce the shrinkage and eventual death of the cell. Gamma-ray irradiation did not induce such a change even at very high doses. It is recommended to have enough information about the quality of HTO ampuled in factory or to find a suitable technique to check it prior to use in the experiments.

ACKNOWLEDGEMENTS

We would like to acknowledge Drs. T.Shiroya and N.Morikawa for the use of the tritium safety cabinet specially designed for HTO handling. Kind cooperation of the staff at the Radio-isotope Centre, University of Tokyo, particularly of Mr.N.Nogawa and Miss M.Ohno is greatly appreciated. This work is supported by a Grant-in-Aid from the Ministry of Education,Science and Culture, Japan.

REFERENCES

1. T. Ito and K. Kobayashi, Mutagenesis in yeast cells by storage in tritiated water. Radiat. Res. 76, 139-144 (1978).
2. M. Hayashibe and S. Katohda, Initiation of budding and chitin-ring. J. Gen. Appl. Microbiol., 19, 23-39 (1973).
3. G. Svihla, F. Schlenk, and J. L. Dainko, Growth and metabolism of yeast cells after lethal X-irradiation. Int. J. Radiat. Biol. 20, 461-474 (1971).
4. C. Baumstark-Khan, L. Schnizler, and H. Rink, Radiation induced formation of giant cells (Saccharomyces uvarum) I. Budding process and chitin ring formation. Radiat. Environ. Biophys. 23, 19-30 (1984).
5. A. Ito and T. Ito, Possible involvement of membrane damage in the inactivation by broad-band near-UV radiation in Saccharomyces cerevisiae cells. Photochem. Photobiol. 37, 395-401 (1983).

NIRS-M-52, pp 81 - 94 , 1985

Analysis at the Molecular Level of Mutations Induced by Tritium, X-rays and Chemical Mutagens

W.R.Lee

Genetics Program
Department of Zoology and Physiology
Louisiana State University
Baton Rouge, LA 70803, U.S.A.

Abstract

Tritium decay can induce mutations by: (1) ionizing radiation and/or (2) transmutation of ^{3}H to ^{3}He with associated energy of excitation and recoil. Ionizing radiation from tritium decay at the different incorporation positions in DNA is assumed to be equivalent throughout the nucleus; therefore, the differences in mutation frequency per tritium decay at different incorporation sites in DNA result from excitation, recoil, and transmutation of ^{3}H to ^{3}He. In previous work, we have detected local or site specific effects of tritium decay by comparing the frequency of sex-linked recessive lethal (SLRL) mutations induced by tritium decay in specific DNA sites of _Drosophila melanogaster_ sperm cells. We have found a site specific effect when tritium decay occurred at the 5 position of cytosine, 8 position of purine or 6 position of pyrimidine. These differences in the site specific mutagenesis can be described only as quantitative differences in

induced mutation frequency because SLRL mutations can be induced by any known mechanism of mutagenesis. Current research is focusing on detecting differences in mutational mechanism by analyzing mutant genes at the molecular level using labeled probes of the locus alcohol dehydrogenase (Adh) to determine molecular changes in DNA of the mutants. We are comparing null mutants induced by x-rays, the alkylating agent N-ethyl-N-nitrosourea (ENU) and decay of tritium incorporated into specific sites of DNA. Current observations are that mutants induced by x-rays have an unusually high frequency of multiple breaks in DNA in relatively short distances near the locus. Most of the X-ray induced mutants were induced through a mechanism of multiple chain breaks in DNA.

Introduction: tritium decay can induce primary lesions in DNA through 1) transmutation of tritium to helium-3 with the associated chemical effects of change in nuclear charge and energy of excitation, 2) recoil of the tritium nuclide, and 3) beta radiation. To determine the frequency at which the primary DNA lesions, resulting from tritium beta decay, become converted into transmissible mutations it is desirable to partition the total mutation frequency into the frequencies that result from the various causes of primary DNA lesions. Recoil of the helium nuclide following tritium decay is not sufficient to break the nuclide out of the surrounding chemical structure. Therefore, the consequence of recoil, energy of excitation, and change in charge of the nuclide are localized to the region of the molecule in which the tritium decay occurs. Consequently, I will describe the combination of transmutation and energy of recoil as a local effect on the DNA molecule; in contrast, the beta particle has a range in water of 0.56μ (1), sufficient to cause primary lesions in DNA through radiation damage to other DNA molecules in the same nucleus.

In previous work (2 & 3) with ^{32}P, we were able to separate the effect of beta radiation from the local effects in sperm cells of Drosophila Melanogaster because the thin diameter of the cylindrical Drosophila sperm

cell (0.4μ) results in most of the ionization from ^{32}P beta decay occurring outside the cell; therefore, by storing Drosophila sperm cells with ^{32}P incorporated into their DNA for a period of three weeks we were able to accumulate the local effects of ^{32}P beta decay without a rise in the mutation frequency resulting from beta radiation.

Local Effect of Tritium Decay: It is not possible to separate the local effect of tritium decay from beta radiation as we did in ^{32}P because a significant amount of the energy from beta radiation resulting from beta decay of tritium will be deposited in the nuclease in which the tritium decay occurs. To separate the local effects of tritium decay from beta radiation we have compared the mutation frequency per decay of tritium incorporated into the different non-exchangeable hydrogen positions of pyrimidine and purine DNA precursors. Beta radiation effects per decay should be equivalent because the mean range of the beta particles from tritium in matter of unit density is 0.56μ (1) which is sufficiently long so that radiation from different points of origin in the nucleus should cancel out in the comparison of tritium decay at different sites of incorporation. Therefore, the net observed difference in the comparison will result from the local effect of decay at a unique incorporation site. The number of tritium disintegrations which have occurred over some time interval can be calculated using the measured level of incorporation at time zero and the decay constant of tritium, Since sensitivity to beta-radiation changes with time as germ cells pass through successive stages in development, it is necessary to hold the stage of germ cell development constant while permitting the accumulation of tritium decay products. To accomplish this we have stored treated spermatozoa in the receptacles of inseminated females. The tritium labeled spermatozoa are allowed to fertilize eggs of Drosophila melanogaster and pro-mutagenic lesions are either repaired or converted into mutations which can be analyzed using the Drosophila test systems for mutations. For comparative effects of disintegrations at specific sites the mutation

frequency per tritium decay is determined.

All of the seven non-exchangeable hydrogen positions on the purines and pyrimidines - 5 and 6 of cytosine, 5-methyl and 6 of thymine, 2 of adenine and 8 of adenine and guanine - have been studied for local position-specific effects in prokaryotes. Dr. Stanley Person and co-workers have summarized their findings that transmutation of tritium in the 5-position of cytosine, the 6-position of thymine and the 2-position of adenine produced mutations over the frequency that could be attributed to beta radiation (4). When reverse mutation or suppressors of the UAG codon were used for mutation detection, 5-labeled cytosine was far more mutagenic than 6-labeled cytosine or thymine (5 & 6). Decay at the 6-position of the pyrimidine produced increases in DNA crosslinkage and single-strand breakage (6). 8-^{3}H-guanosine (also incorporated as 8-^{3}H-adenosine) gave no higher frequency of local effects than that observed with ^{3}H-methyl-thymine (4).

We have used the work in prokaryotes as a guideline in designing our tests and establishing expectations for the Drosophila germ cell mutagenicity studies, although we are somewhat limited in our ability to make direct comparisons with previous work by the (sometimes not altogether clear) differences in sensitivity of the various test systems to different types of genetic damage. It is entirely possible that tritium decay in one position of a nucleotide base may lead primarily to depurination of DNA while decay in another position may cause only transitions; further more, tritium decay in either position may give similar mutation frequencies in the broad spectrum sex-linked recessive lethal test in Drosophila (7). Our previously published methods (8 & 9) have been used to label immature premeiotic gonial cells in male Drosophila by allowing second instar male larvae to feed on media containing the following tritium labeled DNA precursors: (^{3}H-methyl) thymidine, (5-^{3}H) deoxycytidine, (8-^{3}H) deoxyguanosine, (5-^{3}H) uridine, and (6-^{3}H) uridine. Due to biochemical actions <u>in vivo</u>, tritium from labeled guanosine may also be incorporated as (8-^{3}H) adenine. Tritium induced as

5-labeled uridine appears in the DNA only as (5-^{3}H) cytosine, while that from 6-labeled uridine appears in DNA as both (6-^{3}H) cytosine and (6-^{3}H) thymidine.

Upon emergence as adults the males were allowed to mate, thus transferring the labeled spermatozoa to unlabeled females. A sample of eggs was taken immediately from the newly inseminated females and is referred to as having been derived from the non-stored sperm. A second sample of eggs was taken after storage at 25°C of the inseminated females on media that inhibits egg laying (2 & 3). Genetic tests for mutations were conducted on each of the samples using the same test stocks and procedures described in a previous publication (9). The difference between the mutation frequencies from eggs taken before storage and those taken following storage is a measure of the increase in mutation frequency which is due to events which took place during the storage period. This procedure gives us a defined germ cell stage (mature sperm) with a known structure in which we can measure the genetic effects of tritium decay.

The number of tritium disintegrations responsible for the increased genetic damage during storage was determined by first measuring the amount of tritium incorporated per cell, by methods used previously in our studies of the transmutation effects of ^{32}P (2 & 8). Briefly, this method consists of dissecting the seminal receptacles from inseminated females, stretching and staining on a microscope slide and microscopically counting the sperm cells in the receptacles. Receptacles containing some 3000 cells are washed from the microscope slide onto a glass filter with cold trichloroacetic acid to remove any lower molecular weight material and are digested by the method of Mahin and Lofberg (10) in a sealed scintillation vial. To prevent loss of tritiated water the contents of the vial are frozen before opening the vial to add scintillation fluor. Counts per minute corrected to disintegrations per minute (9) and divided by the number of cells per vial give us the number of disintegrations per minute per cell, and from the known decay rate of ^{3}H

the number of ^{3}H disintegrations during the storage period is computed. The mutation frequency per decay is determined by subtracting the mutation frequency before storage (each frequency having been corrected for the appropriate control frequency) and dividing by the number of tritium decays per sperm cell that would have occurred during the time of storage.

All tests were conducted at 25°C except for F_2 and subsequent generations of the 5-^{3}H-dC and 8-^{3}H-dG experiments which are maintained at 28°C. Positive and negative controls with ethyl methanesulfonate and 1% sucrose showed no increase in mutation detection resulted from incubating the second and later filial generations at 28°C rather than 25°C. Lethals were verified by retesting through two successive generations.

We find that tritium introduced in the larval stage on any of the DNA precursors tested is mutagenic. The increase over control of frequencies of loss of sex-chromatin (significant for 5 of the 6 compounds) and sex-linked recessive lethals (significant for all 6 compounds) observed in the non-stored genetic samples results from the combined effects of beta-radiation (both incorporated in germ cell DNA and in surrounding tissues) and position specific tritium decay throughout the maturation of the germ cells. While the finding that internal exposure to tritium is mutagenic is not surprising, it is important to keep in mind the intrinsic mutagenicity of tritium regardless of its specific-site effectiveness.

Results with the sex-linked recessive lethal test (SLRL) for mutations showed a significant storage effects for all but (5-^{3}H)dC; in the latter case an increase was observed, but the storage population was too small to yield significant numbers of mutations. The storage increase in sex-linked recessive lethal frequencies to number of disintegration of tritium incorporated at specific sites in the DNA was normalized to one for ^{3}H-dT. These estimates are listed in Table 1. The differences between estimates for any two compounds represent the differences in their positions' specific decay effects; their radiation effects per disintegration are presumed to be

equivalent.

The compounds are listed in Table 1 in order of increasing mutation frequency per disintegration. Features worthy of note are the following:

1)tritium incorporated at the 5-position of cytosine gives a higher frequency per tritium disintegration than methyl-labeled thymidine or generally labeled deoxycytidine with 70% of the label in the deoxyribose moiety.

2)Equal mutagenic effect was observed whether the tritium was introduced as (5-^{3}H) deoxycytidine or (5-^{3}H) uridine. This is as we would expect since with both precursors the tritium in the stored sperm cell is incorporated into only the cytosine moiety of DNA. Conversion of the 5-labeled uridine to thymidine results in loss of the tritium, and the apparent lack of uridine label is consistent with the amount of RNA in spermatozoa and the rapid turnover of tritium in the RNA pool during the 10 days between labeling gonia and sampling the mature sperm.

3)The most mutagenic positions of disintegration appear to be the 8 of guanine (adenine) and the 6 of cytosine (thymine), i.e., those beta to the glycosidic bond. We would speculate that disintegration at these positions leads to loss of the purine or pyrimidine base. Mutations would be induced during repair at such sites.

TABLE I

TREATMENT COMPOUND	FORM OF ^{3}H INCORPORATION IN DNA	SLRL PER TRITIUM DISINTEGRATION $(X10^{-5})^a$
Deoxycytidine (G-^{3}H)	(G-^{3}H)dC (G-^{3}H)dT	0.64
Deoxythymidine (Me-^{3}H)	(Me-^{3}H)dT	1.45
Deoxycytidine (5-^{3}H)	(5-^{3}H)dC	3.18
Uridine (5-^{3}H)	(5-^{3}H)dC	3.08
Deoxyguanosine (8-^{3}H)	(8-^{3}H)dG (8-^{3}H)dA	4.49
Uridine (6-^{3}H)	(6-^{3}H)dC (6-^{3}H)dT	5.30

[a]Sex-linked recessive lethal frequencies before and after storage were corrected for their appropriate control frequencies and the differences between them was divided by the number of tritium disintegrations during storage.

Molecular Comparison of Altered DNA From Induced Mutants: The above SLRL test provides a means of quantitatively determining that there is a local effect observed as an increase in total mutation frequency if tritium decay occurs at specific sites of DNA; however, the SLRL test is incapable of distinguishing the type of lesion produced by different agents because a sex-linked recessive lethal can be induced by virtually every kind of DNA lesion. To determine the characteristic of the DNA lesion that resulted in a mutation, we are using the methods of recombinant DNA techniques to characterize the mutant alleles. Our approach is to characterize mutants produced by x-ray as characteristic of those induced by ionizing radiation, recognizing that the linear energy transfer of tritium beta decay is higher than that of x-ray. As a contrast to ionizing radiation, we are studying mutants induced by N-ethyl-N-nitrosourea (ENU). The results of our studies of mutants induced by x-rays are complete (11) and tests are in progress to permit comparisons among null mutants induced by x-rays, the alkylating agent N-ethyl-N-nitrosourea (ENU) and decay of tritium incorporated into specific sites of *Drosophila melanogaster* DNA.

The isolation of *Adh* negative mutants is based on their ability to survive exposure to 1-penten-3-ol, while flies that are positive for the ADH enzyme convert the secondary alcohol to a lethal ketone (12 & 13). Genetic complementation tests are performed using deletions with known breakpoints extending to both sides of the *Adh* locus (14, 15, 16 & 17). Large deletions are quickly screened from the rest of the mutants and are not further characterized at present. Mutants which do not fall into this category are maintained in stock over known deletions at this locus.

The initial analysis of the mutants is for the presence or absence of the ADH enzyme which is accomplished by using a modification of the O'Farrell (18) two-dimensional electrophoresis technique. This method is capable of identifying a mutant protein of a single base change or small intragenic deletions which produce a protein within 10% of the normal length. If ADH

protein is detected, alterations in the amino acid sequence can be determined.

The presence of the Adh mRNA is determined by the dot blot technique (19). If no RNA is present, the mutation may be transcriptional. Mutants are then subjected to DNA restriction endonuclease Southern blot analysis (20 & 21). Hybridizations are done by using ^{32}P-labeled genomic clones of Adh (sAC1-4.8kb and sAF2-11.8kb) (22) and ^{32}P-cDNA (23) for the 3′ half of the gene. The sAC1 genomic clone includes approximately 2 kilobases on either side of the Adh structural gene which, with its two introns, is only 1 kilobase in length (24); the sAF2 clone contains approximately 5.4kb on either side of the Adh gene. If DNA and RNA are present with no alterations detected, the ADH deficiency could be classified as a translational mutant.

Of 31 Adh negative mutants induced by x-ray and two spontaneous mutants made available to us for this study, Aaron (25). By classical genetic mapping 23 are classified as deletions. The 10 remaining negative mutants are neither large deletions nor do they show any cytological aberrations of salivary gland chromosome bands. However, the genetic complementation tests and salivary gland chromosome analysis are at least an order of magnitude lower in sensitivity than the techniques used here. Three of these mutants have been lost. We have analyzed the remaining 7 mutants for molecular changes in DNA (11). In summary, mutants nLA73, 74, 80 (3000 rads), mutant nLA2 (1000 rads), mutants nLA248, and 249 (500 rads) and nLA319 (spontaneous) all hybridize with the sAC1 and 3′ half cDNA probes in a RNA dot blot. Only two mutants, nLA80 and nLA249, produce any detectable ADH protein as determined by the two-dimensional electrophoresis technique. The *in vitro* translation results are inconclusive at the present.

Restriction mapping and Southern blotting experiments have revealed some alterations in the DNA of these mutants. Digestion with HhaI and probing with the sAC1 clone gives normal restriction patterns form mutants 73, 74, 80, 249, 319, and 405, while mutant 2 has an additional bp band. Mutant 248

has an additional 450 bp band when digested with HhaI. Using the larger sAF2 probe and digesting with the same enzyme shows mutant 74 is missing a 1200 bp band fragment and has a new 900 bp fragment. This could be the result of a 300 bp deletion internal to two HhaI sites, but must be outside the region covered by the sAC1 probe since these alterations are not seen when using the smaller sAC1 probe. Mutant 319 (spontaneous) has a pattern similar to that of mutant 74. It is missing the same 1200 bp fragment and has the extra 900 bp fragment, but has also additionally 620 and 800 bp fragments. Double digestion with HpaI and HinfI and probing with sAF2 again reveals the extra 1600 bp fragment for mutant 2. This extra fragment is also seen when the shorter sAC1 probe is used. This extra Adh DNA must be inserted somewhere within the region maintained either by the CyO^{nB} balancer chromosome or the deletion 64j. The DNA must have originated from the 4.8 kb region since it hybridizes with the sAC1 probe. However, mutant 319 which again shows the missing and additional fragments when probed with sAF2 after HpaI / HinfI digestion appears normal when probed with sAC1. The alterations for this mutant are outside the 4.8 kb (sAC1) region, but within the 11.8 kb (sAF2) region. The fragments of 643, 893, and 399 bp span the region from the promoter past the 3´ end of the Adh gene. These appear normal in all the mutants analyzed with HpaI / HinfI digestion, at least within the limits of detection. Mutants 74 and 80 are missing a 1400 pb fragment after digestion with HpaI / HinfI. Mutant 248 has all the expected bands, but also additional bands of 520 and 310 bp. The mutant 248 has recently been sequenced in Michael Ashburner´s laboratory, and from personal communication with Dr. Ashburner we have learned that 248 has a 250 base pair duplication within the structural gene that would give a near normal banding pattern for the three bands associated with the Adh locus plus an additional small band as seen in our Southern blot experiments.

If the 23 mutants previously shown to be deletions are added to the 5 mutants showing an abnormal endonuclease Southern blot, at least (28/33) 85% of the mutants are associated with multiple DNA chain breaks.

Literature Cited

1. L. E. Feinendegen, Tritium-Labeled Molecules in Biology and Medicine. Academic Press, N.Y. 1968.

2. W. R. Lee, C. K. Oden, C. A. Bart, C. W. Debney and R. F. Martin, Stability of Drosophila chromosomes to radioactive decay of incorporated phosphorus-32. Genetics 53:807-822(1966).

3. W. R. Lee, G. A. Sega and C. F. Alford, Mutations produced by transmutation of phoosphorus-32 to sulfur-32 within Drosophila DNA. PNAS 58(4):1472-1479(1967).

4. S. W. Person, W. Snipes and F. Krasin, Mutation production from tritium decay: A local effect for (^{3}H)2-adenosine and (^{3}H)6-thymidine decays. Mutation Research. 34:327-332(1976).

5. F. Krasin, S. Person, R. D. Ley and F. Hutchinson, DNA crosslinks, single-strand breaks and effects on bacteriophage T4 survival from tritium decay of (2-^{3}H)adenine, (8-^{3}H)guanine. Journal of Molecular Biology. 101:197-209(1976a).

6. F. Krasin, S. Person, W. Snipes and B. Benson, Local effect for (5-^{3}H) cytosine decays: Production of a chemical product with possible mutagenic consequences. Journal of Molecular Biology 105:445-452(1976b).

7. W. R. Lee, S. Abrahamson, R. Valencia, E. S. von Halle, F. E. Wurgler and S. Zimmering, The sex-linked recessive lethal test for mutagenesis in Drosophila melanogaster. Mutation Research 123:183-279(1983).

8. W. R. Lee, Dosimetry of chemical mutagens in eukaryote germ cells. In Chemical Mutagens (A. Hollaender and F. J. de Serres, Eds.), pp. 117-202. Phlenum Publishing Co., 1978.

9. C. S. Aaron and W. R. Lee, Molecular dosimetry of the mutagen ethyl methanesulfonate in Drosophila melanogaster spermatozoa: Linear relation of DNA alkylation per sperm cell (dose) to sex-linked recessive lethals. Mutation Research 49:27-44(1978).

10. D. T. Mahin and R. T. Lofberg, A simplified method of sample preparation for determination of tritium, carbon-14, or sulfur-35 in blood or tissue by liquid scintillation counting. Anal. Biochem. 16:500-509(1966).

11. M. R. Kelley, Inka P. Mins, Chris M. Farnet, Sherry A. Dicharry and William R. Lee, Molecular Analysis of X-ray Induced Alcohol Dehydrogenase (Adh) Null Mutations in Drosphila melanogaster. Accepted in Genetics.

12. W. Sofer and M. A. Hatkoff, Chemical selection of alcohol dehydrogenase negative mutants in Drosophila. Genetics 72:545-549(1972).

13. J. O´Donnell, L. Gerace, F. Leister and W. Sofer, Chemical selection of mutants that affect alcohol dehydrogenase in Drosophila. II. Use of 1-pentyne-3-ol. Genetics 79:73-83(1975).

14. J. O´Donnell, H. C. Mandel, M. Krauss and W. Sofer, Genetic and cytogenic analysis of the Adh region in Drosophila melanogaster. Genetics 86:553-566(1977).

15. R. C. Woodruff and M. Ashburner, The genetics of a small autosomal region of Drosophila melanogaster containing the structural gene for alcohol dehydrogenase. I. Characterization of deficiencies and mapping of Adh and visible mutations. Genetics 92:117-132(1979a).

16. R. C. Woodruff and M. Ashburner, The genetics of a small autosomal region of Drosophila melanogaster containing the structural gene for alcohol dehydrogenase. II. Lethal mutations in the region. Genetics 92:133-149(1979b).

17. M. Ashburner, C. S. Aaron and S. Tsubota, The genetics of a small autosomal region of Drosophila melanogaster, including the structural gene for alcohol dehydrogenase. V: Characterization of X-ray induced Adh null mutations. Genetics 102:421-435(1982).

18. P. H. O´Farrell, High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021(1975).

19. P. S. Thomas, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proceedings of the National Academy of Sciences. 77:5201-5205(1980).

20. E. Southern, Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98:503-517(1975).

21. G. Smith and M. D. Summers, The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. 109:123-129(1980).

22. D. A. Goldberg, Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. *Proceedings of the National Academy of Sciences* 77:5794-5798(1980).

23. C. Benyajati, N. Wang, A. Reddy, E. Weinberg, and W. Sofer, Alcohol dehydrogenase in Drosophila: Isolation and characterization of messenger RNA and cDNA clone. *Nucleic Acids Research* 8:5649-5667(1980).

24. C. Benyajati, A. R. Place, D. A. Powers, and W. Sofer, Alcohol dehydrogenase gene of *Drosophila melanogaster*: Relationship of intervening sequences to functional domains in the protein. *Proceedings of the National Academy of Sciences* 78:2717-2721(1981).

25. C. S. Aaron, X-ray induced mutations affecting the level of the enzyme alcohol dehydrogenase in *Drosophila melanogaster*: Frequency and genetic analysis of the null enzyme mutants. *Mutation Research* 63:127-137(1979).

NIRS-M-52, pp 95 - 100 , 1985

Dose Rate Effects of Mutation Induction by Tritiated Water in Mouse Leukemia Cells

N.Nakamura[1) , N.Morikawa[2) , N.Nogawa[2) and S.Okada[3)

1) Department of Pathology
Radiation Effects Research Foundation
Hijiyama Park 5-2, Hiroshima 730

2) Radioisotope Center
University of Tokyo

3) Department of Radiation Biophysics
Faculty of Medicine
University of Tokyo
Hongo 7-3-1, Tokyo 113 , Japan

INTRODUCTION

In an assessment of mutagenic potency of tritium, low dose and/or low dose rate study is obviously important but very few such studies were carried out mainly because of the difficulty of handling tritium water which evaporates and contaminates experimental facilities or researchers. In Tokyo University, a "tritium safety clean cabinet" was recently developed which contains tritium trapping system and it became possible to handle as much as 1 Ci of tritium water per experiment. Present study was aimed to clarify dose rate effects of tritium ß-rays

for mutagenesis in cultured mammalian cells at doses mainly below 2 Gy and to compare the results with those obtained after gamma-ray irradiation.

MATERIALS AND METHODS.

Cells and Media. Mouse lymphoma L5178Y cells were cultured in Fischer's medium containing 10% horse serum (denoted as FM10). For colony formation, cells were plated in agar-containing medium (0.18-0.20%) with 15% serum. In order to keep the control mutation frequency to be constantly low, a clonal cell population stored in liquid nitrogen was used for each experiment.

Tritium water treatment. Acute exposure was undertaken by treatment of cells in ice-water bath to prevent possible repairs during irradiation. 40 microliters of tritiated water(5Ci/ml, 185 GBq/ml, Amersham) was taken by a microsyringe and was added to 25 ml of FM10 to make tritium medium of 8 mCi/ml. Logarithmically growing cells were collected by centrifugation at a density of $6x10^7$ cells/ml and 0.2 ml each ($1.2x10^7$ cells) was added to a screw capped glass centrifuge tube containing 2.2 ml of the tritium medium (final tritium concentration was 7.3 mCi/ml). After treatment of 30, 60, 90 and 120 min. in ice-water bath, cells were washed 6 times by centrifugation (1500 rpm x 1 min). The control sample consisted of cells washed immediatedly after addition to tritium medium.

For chronic exposure, 100 mCi of tritiated water was added to 10ml of distilled water. After sterilization by filtration, tritium medium was prepared to give final tritium concentration of 0.25 mCi/ml. $1x10^7$ cells were suspended in 100 ml of the tritium medium and were divided in two 50 ml on day 1. After incubation of cells in air tight culture bottles at 37°C for every 24 hours, more than $5x10^6$ cells for each

duplicate were suspended in 50 ml of freshly prepared tritium medium for further treatment until day 5. The rest of the cells were washed as described above and was used for determination of surviving fraction and mutation frequency.

Determination of surviving fraction and mutation frequency. Immediately after irradiation, a portion of the population was plated for determination of surviving fraction. The rest of the irradiated cells were kept on logarithmic growth by taking more than $1x10^7$ cells in 100 ml of FM10 every day for each sample. Six days after the finish of irradiation, mutation frequency was determined for 6-thioguanine resistance ($6-TG^r$). $8x10^6$ cells per point were suspended in 200 ml of selective medium with melted agar and 50 ml each was placed in a culture bottle for 100 ml. At the same time, about 800 cells were plated in 100ml of non-selective medium for determination of plating efficiency of the population. Final 6-TG concentration was 5 microgram/ml. In order to enumerate resulting colonies after 2 weeks' incubation at 37°C, colony-containing medium was poured into 90mm culture dishes carefully not to introduce air bubbles and colonies were counted by an automated colony counter(Nakamura and Okada,1981). Each set of experiment consisted of duplicated samples including one control and four irradiated groups and experiments were repeated three times for each dose rate.

All the procedures until cell washing were carried out in a safety clean cabinet containing inner box and tritium trapping system developed by us in Radioisotope Center, University of Tokyo. The supernatant of the first wash was measured after appropriate dilutions. The absorbed doses were calculated by the radioactivity assuming water content of the cells to be 70% (Dobson and Kwan,1976).(e.g.,cells in tritium water of 1 mCi/ml receive 0.14 cGy/min).

RESULTS and DISCUSSION

The dose-response curves were almost linear for both irradiation conditions as shown in Fig.1. The slopes were 2.8×10^{-5} and 1.9×10^{-5} induced mutant per survivor per Gy for acute (30-120 min in ice bath) and chronic (1-4 days at 37°C) exposures respectively.

In order to obtain tritium RBE in mutagenesis, the results were compared with those obtained after gamma-ray irradiations of different dose rates(Nakamura and Okada,1982). The comparisons may be realized by two ways, either to compare the doses required to induce certain amount of mutations (e.g., 1×10^{-5} mutant per survivor) or to compare the initial slopes of each dose-response curves. The results are shown in Table 1. For acute exposures, RBE value is close to 1.5 by either method of comparison. For chronic exposures, however, RBE value

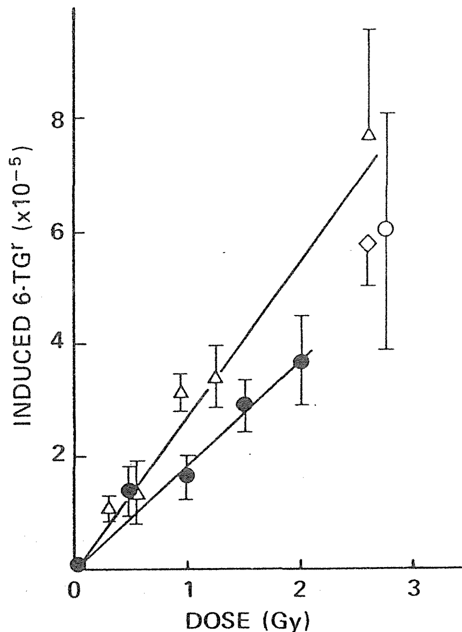


Figure 1. Dose-response curves of 6-thioguanine resistant mutations after acute (△;30 - 120 min in ice bath) and chronic (●;1 - 4 days at 37°C) exposures to tritiated water. (◇;37°C x 2hr, ○ ; 37°C x 48 hr)

largely depends on the method, namely 2.4 for the doses required to give $1x10^{-5}$ mutants per survivor and 4.8 for the initial slopes. This difference is due to the curvilinear nature of chronic gamma-ray dose-response. Although the dose rates of THO and gamma-rays used for the calculations are not identical, it should be mentioned that the THO dose rate is lower than that of gamma-rays and hence RBE values thus obtained would be slightly on the side of underestimation but not on that of overestimation. The increase of RBE value with decreasing the dose-rate is mainly attributable to the refractoriness of dose-rate dependency of tritium beta-rays. This is clearly shown in Fig.2. The mutagenic effects of gamma-rays decrease steadily with decreasing the dose-rate while very slight decrease for tritium. The present study demonstrates importance of low dose-rate studies to estimate biological hazard of tritium ß-rays.

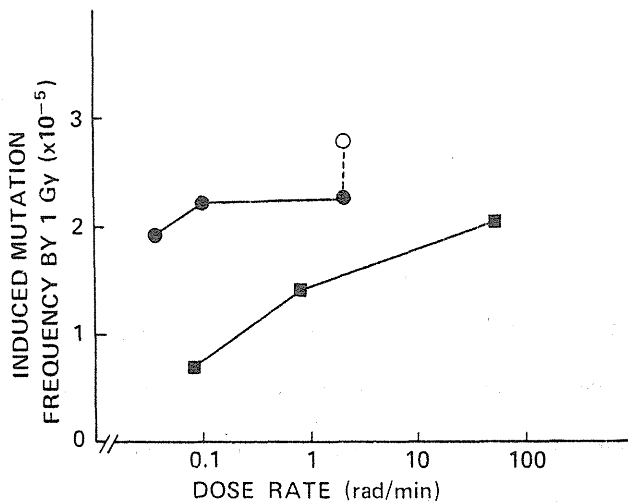


Figure 2. Frequency of the mutation to 6-thioguanine resistance after 1 Gray exposure of tritium ß - or 137-Cs gamma-rays with different dose-rates. ● ;tritium ß -rays, ■ ;137-Cs gamma-rays. Open symbol represents exposure in ice bath and all the other points at 37°C.

Table 1. Calculation of tritium ß-ray RBE of mutations under acute and chronic exposure conditions.

Exposures	Dose required to induce $1x10^{-5}$ mutant frequency		Initial slope of dose response curves($x10^{-7}$/rad)	
Acute gamma	52	) RBE 1.5	1.8	) RBE 1.6
Acute THO	35		2.9	
Chronic gamma	126	) RBE 2.4	0.4	) RBE 4.8
Chronic THO	53		1.9	

REFERENCES.

1.NAKAMURA.N. and S.OKADA, Dose-rate effects of gamma-ray-induced mutations in cultured mammalian cells. Mutation Res.83;127-135(1981)

2.Dobson,R. and C.Kwan, The RBE of tritium radiation measured in mouse oocytes: Increase at low exposure levels. Radiation Res. 66;615-625(1976).

3.NAKAMURA.N. and S.OKADA, Mutation in cultured mammalian cells; Dose and dose-rate effects. Proceedings of the Workshop on Tritium Radiobiology and Health Physics,pp35-45,(1982) Eds.H.Matsudaira T.Yamaguchi, T.Nakazawa and C.Saito, National Institute of Radiological Sciences, Chiba, Japan.

NIRS-M-52, pp 101 - 109 , 1985

Mutation Induction by Tritiated Water and Effects of Some Agents in Mouse Leukemia Cells

I.Furuno-Fukushi and H.Matsudaira

Division of Biology
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

INTRODUCTION

In our previous report, we obtained RBE values of tritium β-rays, 1.5 for cell killing and 1.8 for mutation induction based on the dose-response relationships in cultured mouse leukemia cells after irradiation at 37°C under the condition of cell growth(1).

In this paper, mutation induction following exposure to tritiated water was studied and compared to that following γ-ray exposure. Combined effects of deuterium oxide and some other agents with these radiations were also examined to look for qualitative differences if any of tritium exposure relative to γ-rays. Since,Ueno et al.recently found that the presence of deuterium oxide (D_2O) and 3-aminobenzamide (3AB), an

inhibitor of poly(ADP-ribose) synthetase, during exposure inhibits the dose-rate effects of γ-rays when cell killing was used as an end point(2,3).

MATHERIALS AND METHODS

Details have been described already (1). Outline and some modifications are given below.

Cell culture. Mouse leukemia L5178Y cells were cultured in suspension in Fischer's medium supplemented with 10% horse serum at 37°C. Cells in the logarithmic phase of growth were used.

Gamma irradiation. Cells were irradiated at different distances from a ^{60}Co source to give dose rates of approximately 0.05Gy/hr to 0.4Gy/hr. Irradiation was carried out for a fixed period of 20 hr at 37°C. The cell concentrations at the start of exposure were $1\text{-}3\times10^{5}$/ml. For acute irradiation, the cells were irradiated at room temperature with another ^{60}Co source at a dose rate of 0.3Gy/min.

Treatment with HTO. Cells were treated with different concentrations of HTO (approximately 0.25mCi/ml ~ 2.5mCi/ml)to give dose rates 0.025Gy/hr to 0.25Gy/hr for a fixed time of 20 hr in a tightly capped tube at 37°C. The cell concentrations used were the same as in γ-ray-experiments. The dose rates were estimated by counting radioactivity of the culture medium and using the equation given in ref. 1. After exposure to HTO, cells were washed four times with fresh medium before assay.

Cell survival and mutation. After exposure to γ-rays or HTO, cells were suspended in fresh media. Samples were taken and plating efficiency was determined. After 6 days in culture for mutation expression, the mutation frequency to 6-thioguanine resistance was measured as described.

Drug treatments. Inhibitors at indicated concentrations were present for the duration of exposure to γ-rays or HTO. For acute irradiation experiments, inhibitors were added into the medium just before to 3 hr after irradiation.

RESULTS

Deuterium oxide and gamma-rays. Figure 1 shows changes in the surviving fraction as a function of dose after exposure to γ-rays in the presence or absence of D_2O. A large difference was found in the surviving fraction between acute and chronic irradiation. D_2O at a concentration of 45%(v/v) enhanced cell killing induced by γ-irradiation. The

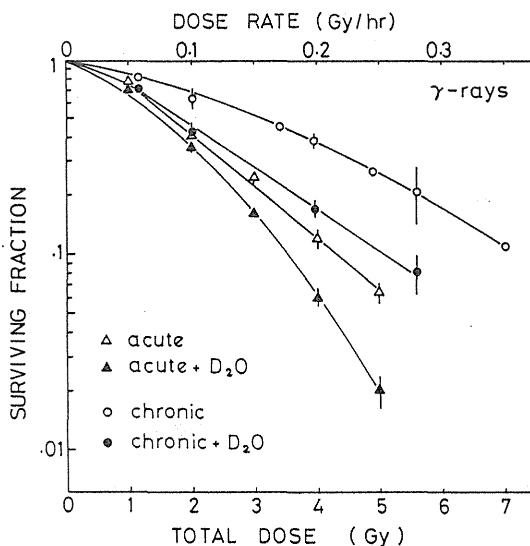


Fig. 1. Changes in the surviving fraction as a function of total dose after acute or chronic γ-irradiation in the presence or absence of 45% D_2O.

survival of cells after chronic irradiation in the presence of D_2O became close to that found after acute irradiation done in its absence.

Figure 2 shows changes in the mutation frequency as a function of total dose after exposure to γ-rays. A large difference was also found in mutation induction between acute and chronic irradiation. D_2O enhanced mutation frequency both for acute and chronic γ-irradiation.

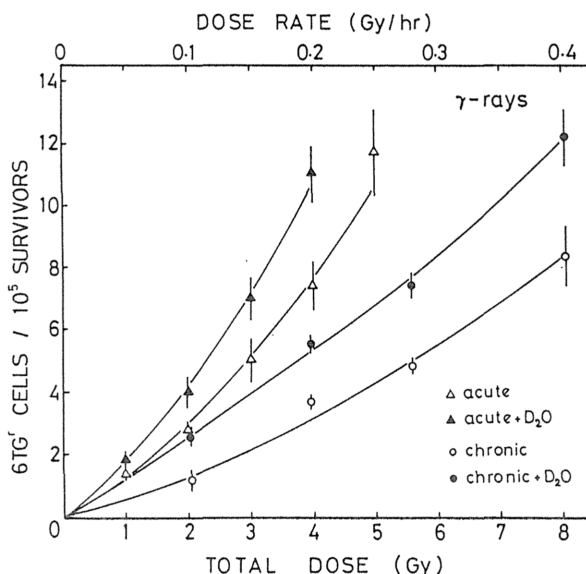


Fig. 2. Changes in the frequency of mutation induction as a function of total dose after acute or chronic γ-irradiation in the presence or absence of 45% D_2O.

Figure 3 shows the correlation of mutation versus surviving fraction, when the mutation frequency was plotted against the logarithm of surviving fraction. The data was taken from Fig.1 and Fig.2. The presence of 45% D_2O caused no change in the survival-mutation correlation from that found after γ-rays alone.This indicates that D_2O enhanced both mutation induction and cell killing of γ-rays to a similar degree.

Deuterium oxide and HTO. Figure 4 shows changes in the

surviving fraction as a function of total dose after exposure

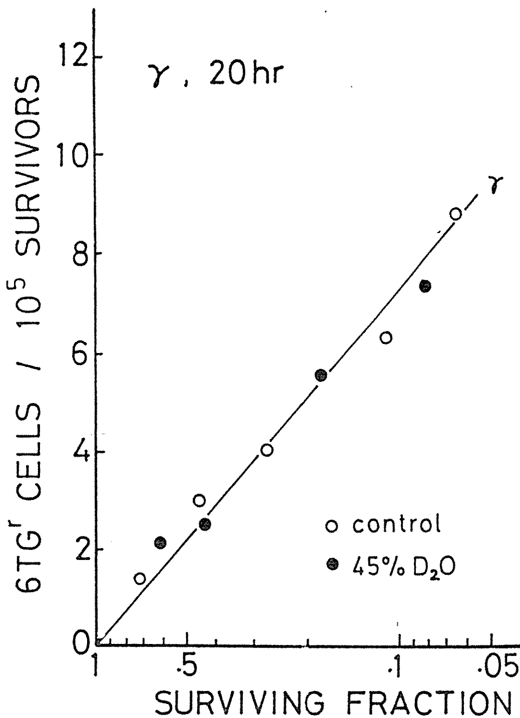


Fig. 3. Survival-mutation correlation after chronic γ-ray irradiation in the presence or absence of 45% D_2O. Data was taken from Fig.1 and Fig.2.

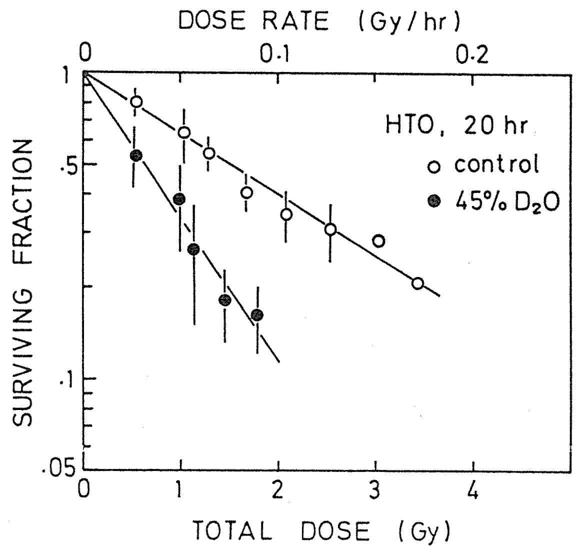


Fig. 4. Changes in the surviving fraction as a function of total dose after exposure to HTO in the presence or absence of 45% D_2O.

to HTO for 20 hr. D_2O enhanced cell killing induced after exposure to HTO to an extent similar to that after γ-rays.

Figure 5 shows changes in the induced mutation frequency after HTO treatment. The frequency of 6-thioguanine resistant cells increased linearly with total dose. D_2O enhanced mutation induced after exposure to HTO.

Figure 6 shows survival-mutation correlation for tritium β-ray irradiation. In the case of HTO, we obtained the same relationship irrespective of the presence or absence of D_2O. The slope of the survival-mutation curve with HTO was , however, steeper than that obtained with γ-rays.

Other inhibitors and gamma-rays. Aphidicolin, an inhibit-

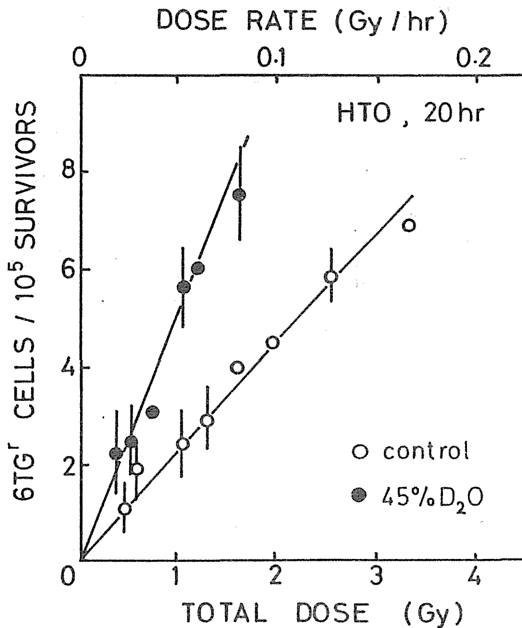


Fig. 5. Changes in the frequency of mutation induction as a function of total dose after exposure to HTO in the presence or absence of 45% D_2O.

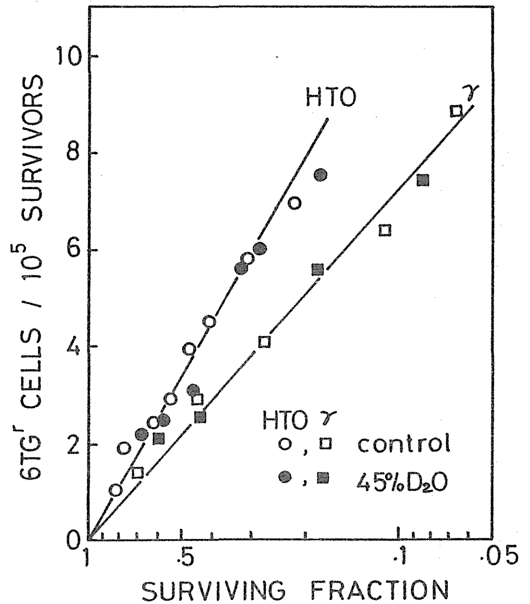


Fig. 6. Survival-mutation correlation after exposure to HTO in the presence or absence of 45% D_2O. Data was taken from Fig.4 and Fig.5.

or of DNA polymerase α, did not affect the mutation frequency, although it suppressed cell growth when present for 20 hours during γ-irradiation at a concentration of 0.2μg/ml(data not shown).

Figure 7 shows changes in the mutation frequency after chronic or acute γ-irradiation in the presence or absence of 3AB. 3AB when given at 10 mM enhanced both cell killing and mutation induced by γ-rays. The enhancement of mutation induction by 3AB was, however, less than that by D_2O.

The effect of combined treatment of tritium with these inhibitors is under examination.

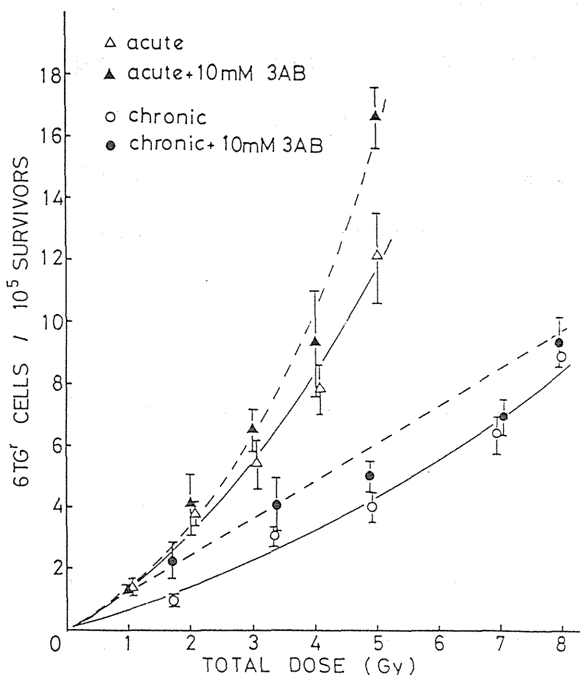


Fig. 7. Changes in the frequency of mutation induction as a function of total dose after acute or chronic γ-irradiation in the presence or absence of 10 mM 3AB.

DISCUSSION

Mutation induction by high LET radiation has been the subject of some experimental investigations: heavy ions (4), neutrons (5), and α particles(6-8). The results indicated that RBE values of high-LET radiations are higher when estimated by mutation induction than by cell killing, although, contradictory findings were also reported(8). This may be due to variations of cell lines, radiation sources and other experimental systems. Results in Fig. 6 support our previous report that RBE value of tritium β-rays estimated by mutation induction is higher than that by cell killing.

Deuterium oxide suppressed the dose rate effects of γ-rays(2,3). D_2O at a concentration of 45% showed almost no

toxic effects on unirradiated cells. At higher concentrations or with longer treatment times, deuterium oxide was very toxic(3) . But we comfirmed that D_2O alone did not induce thioguanine-resistant mutant even at toxic concentrations (data not shown). In summary, our present experiments have shown that deuterium oxide enhances both cell killing and mutations induced by γ-rays and HTO, but dose not change the survival-mutation correlation of these radiations.

ACKNOWLEDGMENT

This work is supported by a special grant for project research on biological effects of tritium from the Science and Technology Agency.

REFERENCES

1. A.M.Ueno, I.Furuno-Fukushi, and H.Mastudaira, Induction of cell killing, micronuclei, and mutation to 6-thioguanine resistance after exposure to low-dose-rate γ-rays and tritiated water in cultured mammalian cells(L5178Y). Radiat.Res. 91,447-456(1982).
2. A.M.Ueno, O.Tanaka, and H.Matsudaira, Inhibition of gamma-ray dose-rate effects by D_2O and inhibitors of poly(ADP-ribose) synthetase in cultured mammalian cells. Radiat.Res. 98,574-582(1984).
3. A.M.UENO and H.Matsudaira, Deuterium oxide inhibition of gamma ray dose-rate effects on growing mouse leukemia cells. This proceedings.

4. R. Cox, J.Thacker, D.T.Goodhead, and R.J.Munson, Mutation and inactivation of mammalian cells by various ionising radiations. Nature 267,425-427(1977).

5. N.Nakamura, S.Suzuki,A.Ito, and S.Okada, Mutation induced by γ-rays and fast neutrons in cultured mammalian cells. Differences in dose response and RBE with methotrexate- and 6-thioguanine-resistant systems. Mutation Res. 104, 383-387(1982).

6. J.Thacker, A.Stretch, and D.T.Goodhead, The mutagenicity of α particles from plutonium-238. Radiat.Res.92,343-352 (1982).

7. G.Iliakis, The mutagenicity of alpha particles in Ehrlich ascites tumor cells. Radiat.Res. 99,52-58(1984).

8. B.J.Barnhart and S.H.Cox, Mutagenicity and cytotoxicity of 4.4-MeV α particles emitted by plutonium-238. Radiat.Res., 80, 542-548(1979).

NIRS-M-52, pp 110 - 126 , 1985

Deuterium Oxide Inhibition of Gamma - Ray Dose-Rate Effects in Growing Mouse Leukemia Cells

A.M.Ueno and H.Matsudaira

Division of Biology
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

Effects of deuterium oxide (D_2O) on cell proliferation and survival were studied in cultured mammalian L5178Y cells under growing conditions after acute and low dose-rate irradiation at about 0.1 to 0.4 Gy/hr of gamma-rays. Cell survival increased with decreasing dose-rates (dose-rate effect). The presence of D_2O at 45% during irradiation at low dose-rates suppressed almost totally the increase in cell survival. Action of various inhibitors on the dose-rate effects was studied in relation to that of D_2O. Among the inhibitors tested, 3-aminobenzamide, theobromine and theophylline, inhibitors of poly(ADP-ribose) synthetase, were found to be effective in eliminating the dose-rate effects, while aphidicolin, 2',3'-dideoxythymidine and

β-arabinofuranosyladenine, inhibitors of DNA polymerase(s), were found to be not effective. The D_2O did not inhibit the radiation-induced increase in poly(ADP-ribose) synthesis as measured by the incorporation of ^{14}C-NAD into the acid insoluble fraction, contrary to 3-aminobenzamide. Growth of irradiated and unirradiated cells was inhibited by D_2O at 45% but not by 3-aminobenzamide at 10 mM, except for treatments longer than 30 hr. Possible mechanisms underlying the inhibition are discussed.

INTRODUCTION

The effect of tritium on living cells has been considered to involve mainly effects due to beta rays. However, isotope effects, transmutation effects and intrāmolecular position effects might play some roles in tritium toxicity. In the course of studies on the responses of cells to tritiated water, we became interested in isotope effects of deuterium (mass of 2) within deuterium oxide (D_2O). Also, relatively low toxicity of tritium prompted us to study the characteristics of damage after low dose-rate exposure.

In general, for sparsely ionizing radiations such as X and γ rays, when the dose-rate is lowered and the exposure time is extended, the biological effect, for example cell killing, of a given dose is reduced. The dose-rate effects are considered to involve among others the repair of sublethal damage taking place during the exposure (1). On the other hand, above a certain concentration, D_2O has been reported to enhance the killing effect of acute X-rays, possibly by interfering with repair from sublethal damage (2).

Purpose of the present study was to investigate a combined effect of D_2O with low dose-rate γ-rays on cultured mammalian cells.

Here, we report that the presence of D_2O during low dose-rate exposure totally inhibits the dose-rate effects of γ rays (3). To understand the inhibitory action of D_2O, effects of various inhibitors on the dose-rate effects and cell proliferation were studied in relation to those of D_2O. From these results, possible mechanisms underlying the inhibition are discussed.

MATERIALS AND METHODS

These procedures have been described (4). Brief descriptions are given below.

Cell culture. Mouse lymphocytic leukemia L5178Y cells were cultured in suspension in Fischer's medium supplemented with 10% horse serum at 37°C. Cells in the logarithmic phase of growth were used.

Gamma irradiation and treatment with inhibitors. The cells were irradiated continuously in a 25-ml plastic culture-flask with a ^{60}Co γ-ray source (50 Ci) in a thermoregulated water bath at 37°C at different distances to give dose-rates of approximately 0.1 to 0.4 Gy/hr for periods from 4.5 to nearly 50 hr. For acute irradiation, the cells were irradiated at room temperature with another ^{60}Co source(3000 Ci) at a dose-rate of 0.3 Gy/min. Inhibitors were added at indicated concentrations from just before to 3 hr after acute irradiation or present during the whole period for low dose-rate irradiation.

Cell survival. At various times after commencement of irradiation, samples were taken and cell survival was determined by using the colony forming technique. The plating efficiencies were 80 - 90 % for unirradiated and untreated controls, 70 - 80 % for unirradiated cells treated with 10 mM 3-aminobenzamide, and 40 - 60 % for unirradiated cells treated with 45 % D_2O. These were determined in every experiment and corrected in calculating the surviving fraction.

Poly(ADP-ribose) synthesis. Permeabilization of cells and assay of poly(ADP-ribose) synthetase activity were carried out essentially according to Edwards and Taylor (5). Irradiated cells were permeabilized in the permeabilizing buffer at pH 7.8 and then incubated for 15 min at 30°C in a reaction mixture containing 0.1 μCi (3.7 kBq) of ^{14}C-NAD. After the incubation, radioactivity of the acid insoluble fraction was measured in a liquid scintillation spectrometer.

Assay of NAD. After washing the irradiated and unirradiated cells (1×10^6 cells/tube) by centrifugation, 1.5 ml of ice-cold 10 % perchloric acid was added to each tube. After 30 min at 2°C, the solution was centrifuged and the pH of the supernatant was adjusted to 7.5 by adding 0.33 M K_2HPO_4-KH_2PO_4 containing 1 M KOH. The $KClO_4$ precipitate was removed by centrifugation. The NAD content of the neutralized acid-soluble fraction was measured by using an alchohol dehydrogenase enzymatic cycling method according to Jacobson and Jacobson (6).

RESULTS

Figure 1 shows the changes in the surviving fraction of the cells as a function of the time of exposure to D_2O administered at various concentrations. Effect of D_2O on cell survival could be detected at 55 % and above. The killing effect of D_2O increased with increasing concentrations of D_2O. At 45 % D_2O-concentration or below, the effect was almost marginal. Therefore, a concentration of 45 % D_2O was chosen for the present experiments.

Figure 2 shows the effect of D_2O on the dose-rate effect of γ-rays. D_2O was added to the culture medium and the cells were irradiated continuously at indicated dose-rates at 37°C. As can be seen in this figure, marked dose-rate effects have been found in the

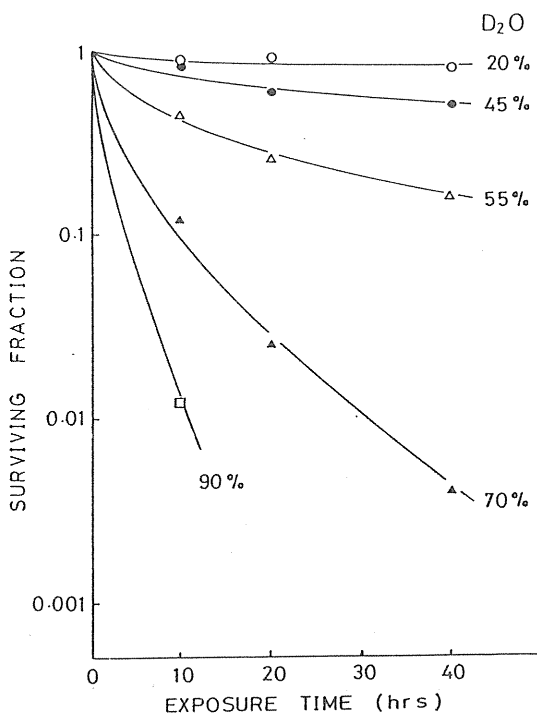


Fig. 1. Changes in the surviving fraction of cells as a function of time of exposure to D_2O administered at 20 % (○), 45% (●), 55 % (△), 70 % (▲) and 90 % (□).

survival of the cells (broken lines). When D_2O at 45 % was present during the exposure to low dose-rate γ rays, the dose-rate effects were almost totally suppressed. The dose-survival curves for irradiations at various low dose-rates were almost superimposable on those obtained after an acute irradiation. The presence of D_2O for a period from just before to 3 hr after the acute irradiation slightly enhanced the killing effect of γ rays.

To understand the inhibitory action of D_2O on the dose-rate effect, the effects of various inhibitors on cell survival after low dose-rate irradiation were studied. Figure 3 summarizes

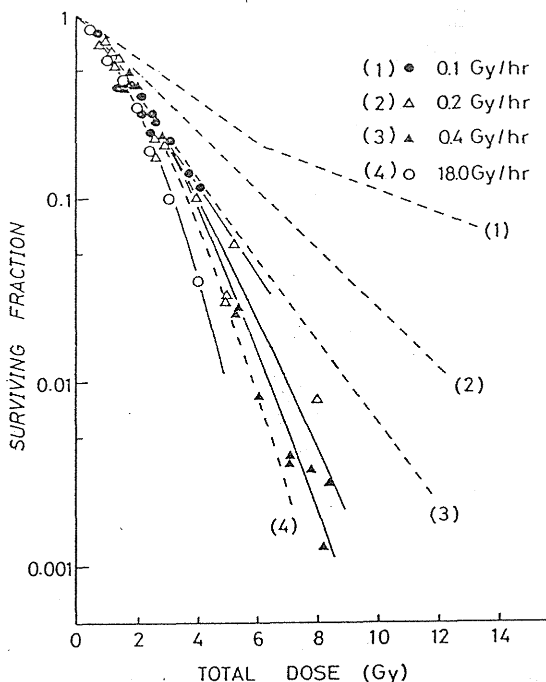


Fig. 2. Changes in the surviving fraction as a function of total dose of γ-rays administered at approximately 0.1 (●), 0.2 (△), 0.4 (▲) and 18 (○) Gy/hr in the presence of 45 % D_2O. The results of irradiation in the absence of D_2O were depicted as broken lines, 0.12 (1), 0.24 (2), 0.48 (3) and 18.0 (4) Gy/hr. Lines were drawn by regression analysis according to the linear-quadratic relationship.

the results for these inhibitors. In these experiments, the irradiation was done for a fixed period of time, i.e., for 20 hr,

at different dose rates. The inhibitors used were 3-aminobenzamide (3AB), theophylline and theobromine, inhibitors of poly(ADP-ribose) synthetase (7,8), and aphidicolin (9), 2',3'-dideoxythymidine (10) and β-arabinofuranosyladenine (11), inhibitors of DNA polymerase(s). These were tested for survival at different concentrations. The figure depicts results at indicated concentrations which were not found to be too toxic for unirradiated cells. The curve for γ

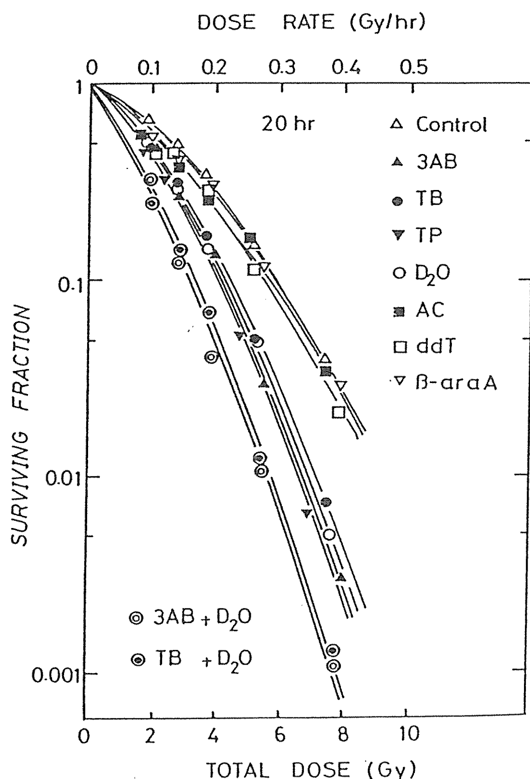


Fig. 3. Changes in the surviving fraction as a function of total dose and dose-rate after irradiation for 20 hr with low dose-rate γ-rays in the presence of various inhibitors. Cells were irradiated in the culture medium containing none (△), 10 mM 3AB (▲), 1 mM theobromine (●), 5 mM theophylline (▼), (0.2 μg per ml) aphidicolin (■), 2 mM dideoxythymidine (□), 12 μM β-arabinofuranosyladenine (▽), 45% D_2O (○), 45 % D_2O + 10 mM 3AB (◎) or 45 % D_2O + 1 mM theobromine (⊙). Each point represents mean of more than two experiments each performed in duplicate. Lines were drawn by regression analysis according to the linear quadratic relationship.

ray alone showed a marked curvature, indicating the dose-rate effects. Inhibitors of DNA polymerase(s) were found to be ineffective in eliminating the dose-rate effects. On the contrary, inhibitors of poly(ADP-ribose) synthetase were found to be very effective in abolishing the dose-rate effects. The extent of the inhibition was similar to that found with D_2O. Combination of 3AB or theobromine with D_2O further enhanced the killing effect of low dose-rate γ rays. 3AB was used for further study on the dose-rate effects. As can be seen in figure 4, the results quite similar to those for D_2O were obtained.

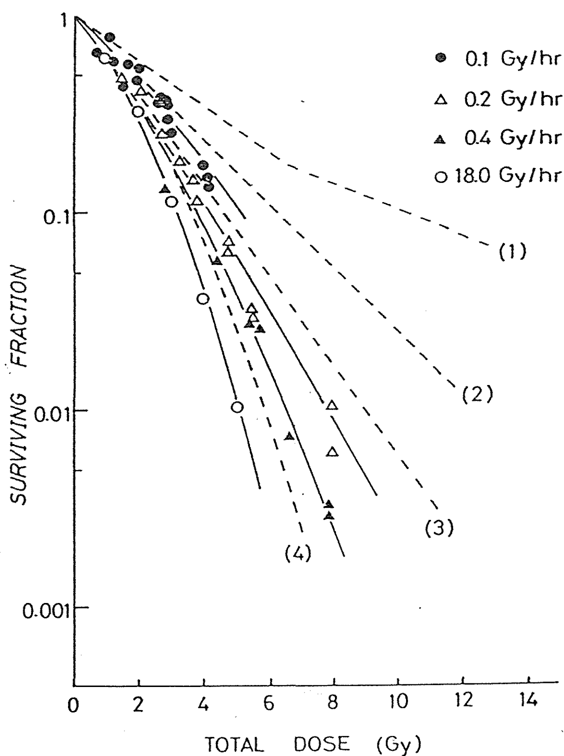


Fig. 4. Changes in the surviving fraction as a function of total dose of γ-rays administered at approximately 0.1 (●), 0.2 (△), 0.4 (▲) and 18 (○) Gy/hr in the presence of 10 mM 3-aminobenzamide. The results of irradiation in the absence of inhibitor were depicted as broken lines, 0.12 (1), 0.24 (2), 0.48 (3) and 18.0 (4) Gy/hr. Lines were drawn by regression analysis according to the linear-quadratic relationship.

To ascertain whether D_2O inhibits an activity of poly(ADP-ribose) synthetase or not, incorporation of ^{14}C-NAD [a precursor of poly(ADP-ribose)] into the acid insoluble fraction of cells was determined. The results are shown in Table I. A dose-dependent increase in the incorporation of ^{14}C-NAD into the acid-insoluble fraction of irradiated cells was completely inhibited by the presence of 3AB. However, the addition of D_2O at 45 % did not affect the radiation-induced increase of poly(ADP-ribose) synthetase activity. Figure 5 shows the changes in the cellular level of NAD as a function of time after 10 Gy of γ rays. Gamma-irradiation induced a transient decrease in the cellular NAD within 30 min, followed by a recovery. The presence of 3AB totally prevented the radiation-induced NAD drop. However, D_2O had no influence on it.

Since D_2O is known to inhibit cell division, possibly by affecting mitotic apparatus, the effects of D_2O and 3AB on the growth of irradiated or unirradiated cells were determined. Figure 6 shows the changes in the number of

TABLE I. Effects of Deuterium Oxide (D_2O) and 3-Aminobenzamide (3AB) on Gamma-Ray Induced Increase of Poly(ADP-ribose) Synthesis in Permeable L5178Y Cells

	Acide-insoluble radioactivity after incubation with ^{14}C-NAD (dpm/µg of protein)			
Inhibitor	Dose in Gy			
	0	25	50	75
None	26.1 ± 1.5*	52.9 ± 5.6	66.3 ± 10.7	95.5 ± 14.4
D_2O(45%)	25.5 ± 1.7	45.3 ± 4.2	61.9 ± 2.9	92.8 ± 10.9
3AB(10mM)	0.9 ± 0.1	0.4 ± 0.2	0.6 ± 0.1	0.4 ± 0.1

* Standard deviation

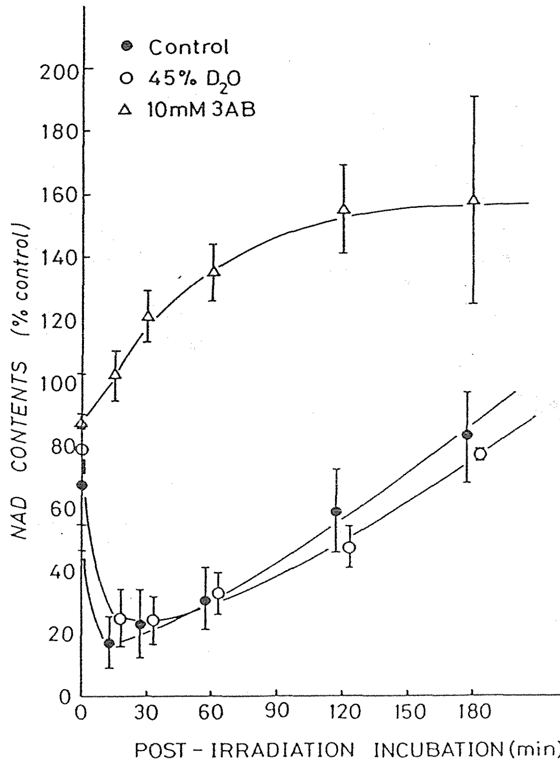


Fig. 5. Changes in the cellular level of NAD as a function of incubation time after 10 Gy of γ-irradiation in the absence (●) or presence of 45 % D_2O (○) or 10 mM 3AB (△).

cells when irradiated continuously in the presence or absence of inhibitors. Irradiation at an increasing dose-rate resulted in an increasing inhibition of the rate of cell growth for the initial 20-30 hr. After this period, the cell number reached a plateau at higher dose-rates (broken lines). The presence of D_2O at 45 % markedly affected the growth of unirradiated cells but did not enhance the inhibition resulting from γ ray exposure (left figure, solid lines). On the other hand, the presence of 3AB at 10 mM affected only marginally the cell growth of both irradiated and unirradiated cells for the initial period, although some inhibition of cell proliferation was seen even in unirradiated cells after treatment for more than 30 hr (right figure, solid lines).

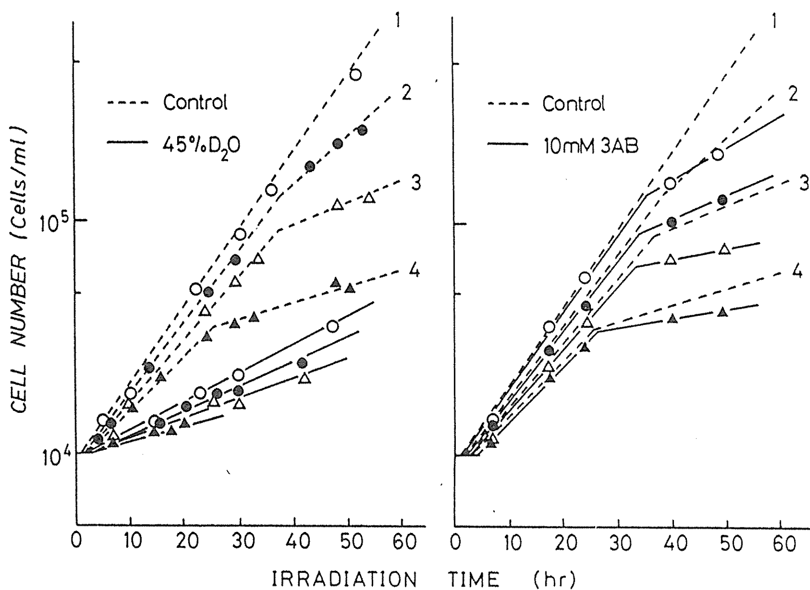


Fig. 6. Effects of deuterium oxide and 3AB on the growth of cells irradiated continuously at 37°C at dose-rates of approximately 0.1 (●), 0.2 (△) and 0.4 (▲) Gy/hr. Unirradiated cells (○) served as control. (a) Irradiation in the absence (----) or presence (——) of 45 % D_2O. (b) Irradiation in the absence (----) or presence (——) of 10 mM 3AB.

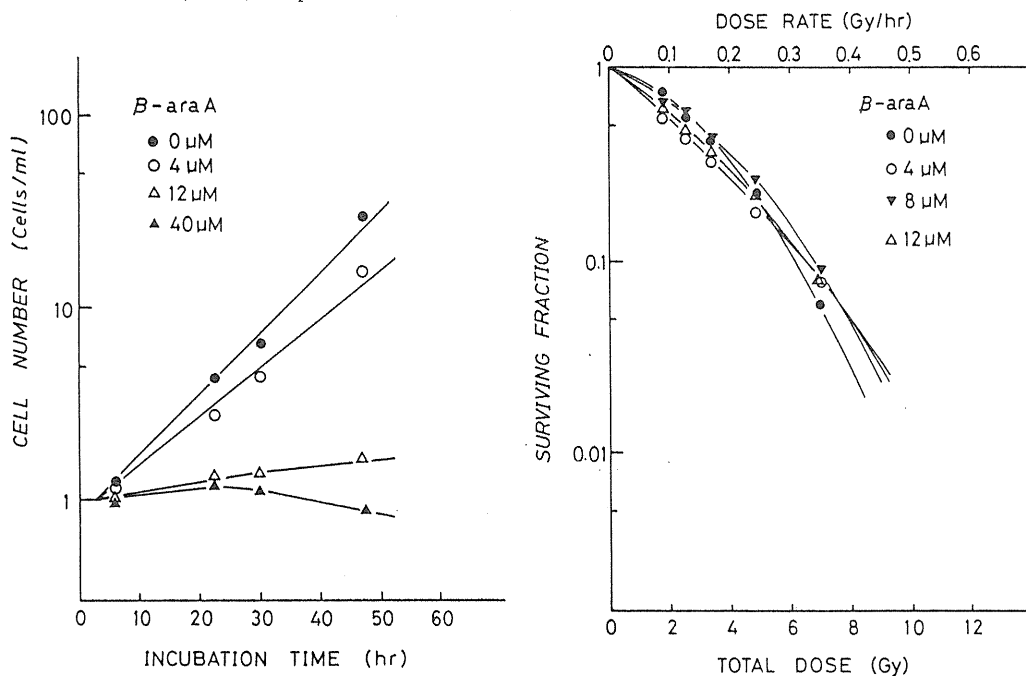


Fig. 7. Effects of β-arabinofuranosyladenine on the growth of unirradiated cells (a) and the survival following low dose-rate γ-rays (b).

Figure 7 shows the effects of β-arabinofuranosyladenine (β-araA), an inhibitor of DNA polymerase α and β, on the growth of un-irradiated cells (left figure) and the survival after irradiation (right figure). β-araA at 12 μM totally inhibited cell growth but did not affect radiation-induced cell killing. The same results were obtained in experiments using aphidicolin, an inhibitor of DNA polymerase α.

Figure 8 shows a summary of the experimental results presented here. It can be seen that there is no correlation between the elimination of dose-rate effects and the inhibition of cell growth. This result indicates that inhibition of cell proliferation during irradiation is not a major factor in eliminating the dose-rate effects.

SUMMARY OF EXPERIMENTAL RESULTS

Inhibitors	Elimination of dose-rate effects	Inhibition of growth
D_2O : 45%	+	+
3-Aminobenzamide : 10mM	+	—
Aphidicolin : 0.2μg/ml	—	+
β-Arabinofuranosyl-adenine : 12μM	—	+

Fig. 8. Summary of the effects of various inhibitors on the dose-rate effects and cell growth.

DISCUSSION

Deuterium oxide and inhibitors of poly(ADP-ribose) synthetase were very effective in inhibiting the dose-rate effects of γ-rays. Recent studies have implicated poly(ADP-ribose) in the repair of DNA damage following treatment with alkylating agents and

ionizing radiation (12,13,14,15). The results presented here support the inference that poly(ADP-ribose) synthesis may be involved in the repair of lethal damage following exposure to low dose-rate γ-rays.

More recently, inhibitors of poly(ADP-ribose) synthesis have been reported to enhance X-ray-induced killing of log-phase Chinese hamster cells by inhibiting the repair of potentially lethal damage. The repair of sublethal damage was not inhibited. A partial difference in the action of poly(ADP-ribose) synthetase inhibitors and that of hypertonic buffer or D_2O was also indicated (16,17). The contribution of repair from sublethal versus potentially lethal damage in the dose-rate effects of low-LET radiations therefore needs further study.

The present paper has shown that D_2O did not interfere with radiation-induced increase of poly(ADP-ribose) synthesis although 3AB completely inhibited it. The action of D_2O may be not common and more complex than that of 3AB. There have been many reports concerning the mechanisms underlying the action of D_2O: Prolongation of the life times of the activated oxygen species (18,19), stabilization of macromolecules and membranes (20,21), inhibition of histone synthesis (22), inhibition of cell proliferation (23,24), induction of increased frequency of chromosomal breakage (25).

Finally, as far as we have examined, the effects of D_2O on L5178Y cells have been detectable at 20% in concentration or above. In D_2O at 20 %, the relative number of ^2H and ^1H atoms is 1 over 4. On the other hand, in tritiated water at 1 mCi/ml, the relative number of ^3H and ^1H atoms is calculated to be 1 over 3.13 X 10^6. Such tremendous difference clearly indicates that the isotope effect of tritium is overwhelmed by radiation effects.

ACKNOWLEDGMENTS

This work was supported by a grant for project research on biological effects of tritium from the Science and Technology Agency and in part by a grant-in-aid from the Ministry of Education,Science, and Culture,Japan.

REFERENCES

1. E. J. Hall, Lethal, potentially lethal, and sublethal radiation damage, and the dose-rate effect. In Radiobiology for the Radiologist, pp. 129-169. Harper & Row, Hagerstown, MD, 1978.

2. E. Ben-Hur and E. Riklis, Deuterium oxide enhancement of Chinese hamster cell response to gamma radiation. Radiat. Res. 81, 224-235 (1980).

3. A. M. Ueno, I. Furuno-Fukushi, and H. Matsudaira, Induction of cell killing, micronuclei, and mutation to 6-thioguanine resistance after exposure to low dose-rate γ rays and tritiated water in cultured mammalian cells (L5178Y). Radiat. Res. 91, 47-456 (1982).

4. A. M. Ueno, O. Tanaka, and H. Matsudaira, Inhibition of gamma ray dose-rate effects by D_2O and inhibitors of poly(ADP-ribose) synthetase in cultured mammalian L5178Y cells. Radiat. Res. 98, 574-582 (1984).

5. M. J. Edwards and A. M. R. Taylor, Unusual levels of $(ADP\text{-}ribose)_n$ and DNA synthesis in ataxia telangiectasis cells following γ-ray irradiation. Nature (London) 287, 745-747 (1980).

6. E. L. Jacobson and M. K. Jacobson, Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells. Archiv. Biochem. Biophys. 175, 627-634 (1976).

7. M. R. Purnell and W. J. D. Whish, Novel inhibitors of poly-(ADP-ribose) synthetase. Biochem. J. 185, 775-777 (1980).

8. K. Ueda, M. Kawaichi, and O. Hayaishi, Poly(ADP-ribose) synthetase. In ADP-ribosylation Reactions. Biology and Medicine (O. Hayaishi and Ueda, Eds), pp. 117-155. Academic Press, New York, 1982.

9. M. Ohashi, T. Taguchi, and S. Ikegami, Aphidicolin: A specific inhibitor of DNA polymerases in the cytosol of rat liver. Biochem. Biophys. Res. Commum. 82, 1084-1090 (1978).

10. M. R. Atkinson, M. P. Deutscher, A. Kornberg, A. F. Russell, and J. G. Moffatt, Enzymatic synthesis of deoxyribonucleic acid. XXXIV. Termination of chain growth by a 2',3'-dideoxy-ribonucleotide. Biochemistry 8, 4897-4904 (1969).

11. G. Iliakis, Effects of β-arabinofuranosyladenine of the growth and pepair of potentially lethal damage in Ehrlich ascites tumor cells. Radiat. Res. 83, 537-552 (1980).

12. C. J. Skidmore, M. I. Davies, P. M. Goodwin, H. Halldorsson, P. J. Levis, S. Shall, and A-A. Zia'ee, The involvement of poly(ADP-ribose) polymerase in the degradation of NAD caused by γ-radiation and N-methyl-N-nitrosourea. Eur. J. Biochem. 101, 135-142 (1979).

13. N. Nduka, C. J. Skidmore, and S. Shall, The enhancement of cytotoxicity of N-methyl-N-nitrosourea and of γ-radiation by inhibitors of poly(ADP-ribose) polymerase. Eur. J. Biochem. 105, 525-530 (1980).

14. D. Creissen and S. Shall, Regulation of DNA ligase activity by poly(ADP-ribose). Nature (London) 296, 271-272 (1982).

15. S. Shall, ADP-Ribose in DNA Repair. In ADP-ribosylation Reaction. Biology and Medicine (O. Hayaishi and K. Ueda, Eds), pp. 477-520. Academic Press, New York, 1982.

16. E. Ben-Hur, H. Utsumi, and M. M. Elkind, Inhibitors of poly-(ADP-ribose) synthesis enhance X-ray killing of log-phase Chinese hamster cells. Radiat. Res. 97, 546-555 (1984).

17. E. Ben-Hur, H. Utsumi, and M. M. Elkind, Potentially lethal and DNA radiation damage: Similarities in inhibition of repair by medium containing D_2O and by hypertonic buffer. Radiat. Res. 84, 25-34 (1980).

18. B. H. J. Bielski and E. Saito, Deuterium isotope effect on the decay kinetics of perhydroxyl radical. J. Phys. Chem. 75, 2263-2266 (1971).

19. P. B. Merkel, R. Nilsson, and D. R. Kearns, Deuterium effects on singlet oxygen lifetimes in solutions. A new test of singlet oxygen reactions. J. Am. Chem. Soc. 94, 1029 (1972).

20. S. Lewin, The use of deuterium oxide for the detection of water-binding of helical structures in nucleic acids and in poly-nucleotides. Arch. Biochem. Biophys. 115, 62-66 (1966).

21. E. V. Obberghen, P. O. Meyts, and J. Roth, Cell surface receptors for insulin and human growth hormon. Effect of microtuble and microfilament modifiers. J. Biol. Chem. 251, 684-6851 (1976).

22. T. R. Henderson, D. M. Dacus, and R. F. Henderson, Deuterium isotope effects on histone synthesis in avian erythroblasts. Arch. Biochem. Biophys. 122, 599-604 (1967).

23. E. F. Oakberg and A. M. Hughes, Deuterium oxide effect on spermatogenesis in the mouse. Exp. Cell Res. 50, 306-314 (1968).

24. E. G. Sideris, R. Mukherjee, and V. Vomvoyanni, Effect of deuterium water on the mitotic cycle, the deoxyribonucleic acid stability, and the frequency of radiation-induced chromosome aberrations in barley. Radiat. Res. 61, 457-467 (1975).

25. H. Joenje, A. B. Oostra and A. H. Wanamarta, Cytogenetic toxicity of D_2O in human lymphocyte cultures. Increased sensitivity in Fanconi7s anemia. Experimentia 39, 782-784 (1983).

NIRS-M-52, pp 127 - 135, 1985

Malignant Cell Transformation Induced by Tritiated Water in Golden Hamster Embryo Cells

O.Nikaido and F.Suzuki

Division of Radiation Biology
Faculty of Pharmaceutical Sciences
Kanazawa University
Kanazawa-shi 920, Japan

ABSTRACT

The transformation frequency in GHE cells has been found to increase significantly with increasing doses of X-rays up to 100 rad and to reach a plateau at doses over 200 rad. This enabled us to assess transformational effects of relatively low doses of tritiated water.

The transformation frequency in cells treated with HTO at ice-cold temperature was higher than that in cells treated at 37° C and eventually reached the same level as that found in cells irradiated with X-rays at a dose-rate of 75 rad/min. These results suggest that DNA repair taking place in cells during HTO treatment reduces the extent of DNA damage leading to malignant transformation.

INTRODUCTION

The studies of transformational effects of ionizing radiation at low doses are necessary particularly as a basis to estimate the cancer risk in exposed human population. On the other hand, with drastic expansion of nuclear industry and fusion research in near future, increased chances of exposure to low levels of radionuclides such as tritiated water (HTO), an ultimate form of tritium in the environment, may be expected. The biological effects of HTO such as cell killing and mutation induction have been reported recently (1). However, transformational effects of HTO in vertebrate cells have not been fully examined yet. Normal embryonic cells derived from golden hamster and established mouse cell line of C3H 10T1/2 have been frequently used as quantitative system for determining transformational effects of ionizing radiation and environmental mutagens. Of these , golden hamster embryo (GHE) cells at very early passage have been shown especially suitable for the quantitative assessment of transformational effects of X-rays at doses lower than 100 rad (2).

In this preliminary study, the GHE cells either irradiated with various doses of X-rays or treated with HTO for various intervals were inoculated into plastic dishes and incubated in CO_2- incubator at $37^{o}C$ for 11 days. The resulted colonies were examined under a dissecting microscope and morphologically altered colonies classified according to the modification of Borek's criteria (3) were counted.

The transformation frequencies in cells treated with HTO

at 37° C were lower than those in cells irradiated with X-rays at a dose rate of 75 rad/min. However, the cells exposed to HTO in ice showed the same level of transformation frequencies as did the cells irradiated at 75 rad/min of X-rays. These results suggested that DNA repair acting in the cells during HTO treatment at 37° C reduced transformation frequencies below the level which was expected from HTO treatment of the cells in ice-cold condition.

MATERIALS AND METHODS

Cell culture. Golden hamster embryo (GHE) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Nissui Seiyaku Co., Tokyo) supplemented with 10 % fetal bovine serum (M.A. Bioproducts Co., Walkersville, Md), penicillin G (100 units/ml) and kanamycin sulfate (100 μg/ml). The methods for obtaining GHE cells were essentially the same as those described previously (2). Briefly, whole golden hamster embryos at 13 to 14th day of gestation were minced with scissors and treated with 0.5 % trypsin solution to disaggregate the cell clumps. After centrifugation, cell pellet was suspended into the medium and inoculated into 75 cm^2 plastic flasks at a high cell density. The cells incubated for 3 days were trypsinized and stored in liquid nitrogen. All experiments were performed with the primary or secondary culture initiated from this frozen stock.

X-Irradiation. The cells were irradiated with X-rays generated from Shimazu Shin-Ai-Go (182 kVp, 15 mA, 0.9 mm Cu + 0.5 mm Al filter) at a dose-rate of 75 rad/min. The exposure doses were measured using a Victoreen condenser chamber

(Victoreen Instruments Co., Cleveland, Ohio). The absorbed doses were calculated with conversion factor presented in ref (4) for relevant half value layer of X-rays. The cells exponentially growing in a 25 cm^2 plastic flask at 37° C were irradiated with X-rays at room temperature.

HTO treatment. Exponentially growing GHE cells in a 25 cm^2 plastic flask were treated with 4 ml of culture medium containing HTO at a concentration of 10 mCi/ml for various times up to 150 min at 37° C. On every experiment, HTO-containing culture medium was collected immediately before and at the end of the exposure, and the radioactivities were determined using a liquid scintillation counter. The radioactivity contained in the medium after treatment tended to be lower than that in the initial culture medium, although the amount of lost activity is not correlated with the length of exposure time (This decrease could result from the dilution by the cold medium remained in the flask). Therefore, we used the radioactivity of HTO-containing medium collected at the end of exposure to calculate the dose of β-rays from HTO.

If the average energy of tritium β-rays is 5.69 keV and the distribution of HTO in the cells is uniform, then the dose rate in rad/min is geven by

mCi/ml medium at the end of exposure x 0.202 x 0.7

where 0.202 is the conversion factor and 0.7 is the assumed water content of the cells instead of 0.8 adopted in ref(1).

To determine the effects of low dose-rate radiation, the cells were also treated with 10 mCi/ml of HTO for vairous times in ice-cold condition. After treatment with HTO-containing medium, cells were washed five times with 10 ml of fresh medium.

Culture flasks were refed and placed in a 37° C incubator or in an ice-cold water bath over the time of the longest exposure.

Transformation assay. This has been described in detail (2). GHE cells cultured for 2 days were irradiated with 5000 rad of X-rays (182 kVp, 15 mA, no filter) and inoculated into 60 mm plastic dishes in 4 ml of complete medium at a density of 2.5 x 10^3 cells/ml (feeder cell inoculation). After incubation overnight, 1 ml of cell suspension containing 400 X-irradiated or HTO-treated cells were added to each dish (target cell inoculation) and incubated in a humidified CO_2-incubator at 37° C for 11 days. Then, the dishes were rinsed with phosphate-buffered saline, and the cells were fixed with ethanol and stained with 3 % Giemsa solution. The morphology of colonies was carefully examined under a dissecting microscope. The colonies showing malignant characteristics such as piling-up, criss-cross of the cells and high cell density were discriminately counted as previously described (2). Transformation frequency was determined by dividing the number of malignant colonies by the total number of colonies counted. More than 5,000 colonies were counted for each dose group per experiment.

RESULTS AND DISCUSSION

Typical results obtained from the cells treated with HTO at 37° C for various times are shown in Table 1. In this series of experiments, the doses of HTO β-rays given to the cells differed slightly from experiment to experiment, since the

doses of β-rays from HTO were determined from the radioactivity contained in the medium at the end of the exposure as mentioned in MATERIALS AND METHODS. Furthermore, spontaneous transformation frequencies in untreated or unirradiated cells slightly differed in every experiment, probably due to culture conditions employed. We, therefore, pooled all the data from more than 7 independent experiments and calculated the transformation frequencies.

Transformation of golden hamster embryo cells following irradiation with HTO β-rays.

Exposure to 8.51 mCi/ml HTO (min)	Dose[1] (rad)	No. of colonies counted	No. of colonies per dish (Mean ± SE)	Surviving[2] fraction (Mean ± SE)	No. of transformed colonies	Transformation[3] frequency
0	0	6,248	79.1 ± 0.7	1.0	1	1.60×10^{-4}
10	12.0	6,251	78.1 ± 0.9	0.99 ± 0.01	2	3.20×10^{-4}
20	24.1	6,304	78.8 ± 0.8	1.00 ± 0.01	2	3.17×10^{-4}
40	48.1	5,786	74.2 ± 0.7	0.94 ± 0.01	3	5.18×10^{-4}
150	180.5	5,263	65.8 ± 0.8	0.83 ± 0.01	3	5.70×10^{-4}

1) Dose rate in rad/min = mCi/ml medium at the end of exposure X 0.202 X 0.7

2) Surviving fraction = $\frac{\text{Number of colonies per dish treated}}{\text{Number of colonies per dish untreated}}$

3) Transformation freqency = $\frac{\text{Number of transformed colonies}}{\text{Number of colonies counted}}$

The surviving fraction of GHE cells irradiated with X-rays decreased exponentially with doses from 100 to 400 rad, although no significant decrease was observed in surviving fraction of cells irradiated with less than 100 rad (shoulder region in the survival curve) as shown in Figure 1. The survival curve for cells treated with HTO in ice-cold condition appeared similar to that for cells irradiated with X-rays at a dose rate of 75 rad/min. However, considerable increase was noticed in the surviving fraction of cells treated with HTO at 37° C compared to that of cells treated in ice-cold condition.

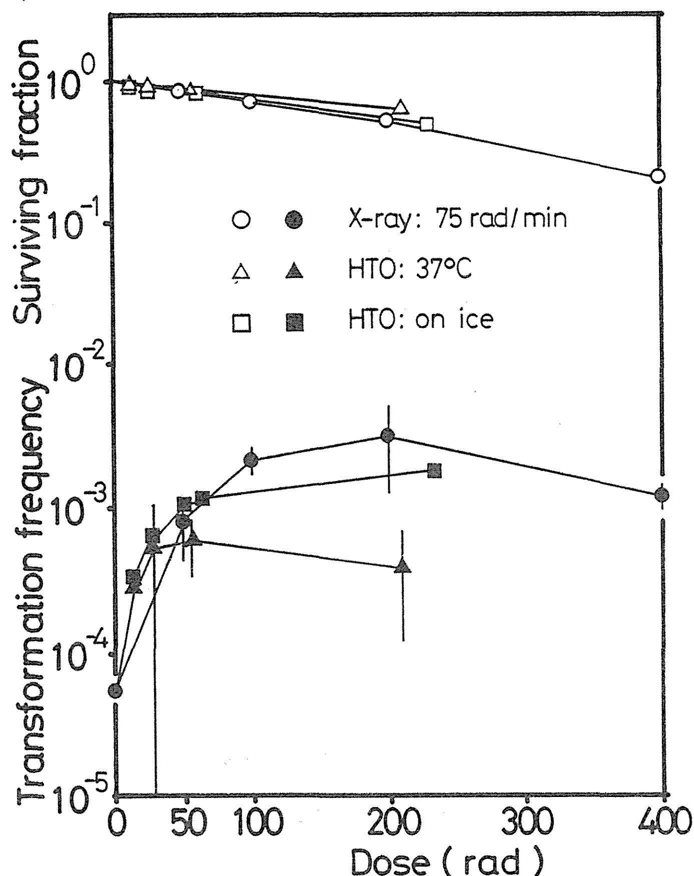


Figure 1. Induction of transformation in golden hamster embryo cells irradiated with X-rays or treated with HTO.

On the other hand, transformation frequency in X-irradiated cells increased with increasing doses up to 200 rad and decreased thereafter. The transformation frequencies in cells treated with HTO at 37° C eventually reached its maximal value when incubated for 40 min. However, transformation frequency in cells treated with HTO in ice-cold condition increased with increasing times of treatment up to 150 min (corresponding to a total dose of 232.4 rad of β-rays) and reached to almost the same level as that found in X-irradiated cells. It is noteworthy that both the cells irradiated acutely with X-rays and the cells treated with HTO in ice gave almost

the same transformation frequencies, although the results under the latter condition are still preliminary. Remarkable differences found in the transformation frequencies between cells treated with HTO at 37° C and at ice-cold temperature suggest that DNA repair might play a role in reducing the amount of DNA damage leading to transformation of GHE cells.

Finally, apprecibale increase in transformation frequencies was observed even when total doses of less than 100 rad of X- or β-rays were given. Thus, the GHE cell system was revealed to be highly sensitive to detect the transformational effects of low doses of ionizing radiation. Further experiments on dose-rate effects of X-irradiation at 37° C and at ice-cold temperature are necessary to obtain RBE of tritium in terms of malignant cell transformation. The experiments along this line are now under way in our laboratory.

REFERENCES

1. A. M. Ueno, I. Furuno-Fukushi and H. Matsudaira, Induction of cell killing, micronuclei, and mutation to 6-thioguanine resistance after exposure to low dose-rate rays and tritiated water in cultured mammalian cells(L5178Y). Radiation Res., 91:447-456(1982).
2. M. Watanabe, M. Horikawa and O. Nikaido, Induction of oncogenic transformation by low doses of X-rays and dose-rate effect. Radiation Res., 98:274-283(1984).
3. C. Borek and E. J. Hall, Transformation of mammalian cells in vitro by low doses of X-rays. Nature, 243:450-453(1973).
4. In "Physical aspects of Irradiation", ICRU Report, 106: 1962, pp1-12, National Bureau of Standards Handbook 85 (1964).

NIRS-M-52, pp 136 - 145 , 1985

Malignant Cell Transformation Induced by Tritiated Water in Mouse 10T1/2 Cells

T.Yamaguchi, M.Yasukawa, T.Terasima and H.Matsudaira

National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

Cultured cells in confluency were irradiated with either β-rays from HTO or ^{60}Co γ-rays. The duration of exposure was 20 hr throughout the experiments. The total dose was varied by changing the dose-rate. Temperature during the 20 hr was either 4°C or 37°C. Dose-response relationships were investigated for colony-forming ability and transformation frequency.

The dose-survival and dose-transformation curves for γ-irradiation at 4°C were nearly the same as those obtained after a single acute X-irradiation (0.5 Gy/min). When γ-irradiation was administered at 37°C, both the lethality and transformation -induction were lower than those after the corresponding doses

---

This work was supported by a grant for project research on biological effects of tritium from the Science and Technology Agency, Japan

at 4°C, indicating the existence of repair from sublethal and subtransformational damages during irradiation at 37°C. The same effect of temperature was observed in the case of HTO exposure. The effect per Gy, however, was higher for the β-rays in comparison with that for the γ-rays in both the responses: Resulting relative biological effectivenesses (RBE's) of tritium β-rays were 1.58 (4°C-irradiation) or 1.56 (37°C) at D_0 for lethality, and 1.5-1.8 (4°C) or 1.4-1.8 (37°C) for cell transformation within a dose range of 0 to 6 Gy.

INTRODUCTION

To predict either the severity or the probability of the deleterious effects on health resulting from irradiation, the International Commission on Radiological Protection (ICRP) has introduced a quantity, called dose equivalent, that correlates particularly with the delayed stochastic effects (1). Then the quality factor (Q) should be based on the relative biological effectiveness (RBE) either for carcinogenesis or for mutation -induction by the radiation in question. The RBE values of tritium compared with X- or γ-radiation have been reported to lie between 1 and 2 for the mutation-induction in bacteria (2), yeast (3), silkworm (4), mice (5) and L5178Y cells (6, 7). On the other hand, there have been no available data on the RBE value of tritium for the carcinogenesis. Thus we attempted to obtain it using the mouse 10T1/2 cells as an experimental system for the detection of malignant transformation *in vitro*.

MATERIALS AND METHODS

The cell line used was C3H 10T1/2 kindly provided by Dr. C. Heidelberger. The cells were grown in Eagle's basal medium (BME; GIBCO Labo., NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (Granite Diagnostics, NC, U.S.A.), 3,3',5-triiodo-L-thyronine (1×10^{-8}M; Sigma, MO, U.S.A.), insulin (100 μIU/ml; Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml). Radiation was always administered to 11th day cultures in the plateau phase of growth. The detailed procedures for survival and transformation assays were the same as those described elsewhere (8), except that the pre-irradiation cultures were initiated with 6.5×10^4 cells per culture flask of 25 cm^2 of growth area (No. 3013, Falcon, CA, U.S.A.). Since the quality of serum markedly influenced the frequency of radiation-induced transformation (8), the same batch of fetal calf serum (Lot No. 10093) was used throughout the experiments, at least in the pre-irradiation culture and for the first 10 days of assay culture. Newborn calf serum (Flow Labo., N.S.W., Australia; Lot No. 29121824 throughout the experiments) was used thereafter in place of fetal calf serum and the assay culture was continued until 8 weeks with weekly renewal of the medium.

Typing of transformed foci was carried out by making reference to the description of Reznikoff _et al_. (9). Since the soft agar colonies were produced exclusively by type III clones (10), only the type III foci were scored as transformed foci. The tumorigenicity of the clones was also evidenced by their malignant growth after inoculation into syngeneic mice in an immunosuppressed condition (10).

The frequency of transformation was calculated by applying the formula of Poisson distribution to the results and expressed as the ratio of the number of transformed foci to the number of surviving colonies.

Low-dose-rate γ-irradiation was done with a ^{60}Co source (50 Ci) in a thermoregulated water bath at either 4°C or 37°C at different water-distances to give a required dose-rate (7).

HTO-treatment was carried out by adding a required amount of HTO (5 Ci/ml; Radiochemical Centre, Amersham, U.K.) into a tightly sealed culture flask which was then maintained at either 4°C or 37°C during the irradiation period. At the end of irradiation the cells was washed well with a fresh BME and trypsinized for survival or transformation assay. The dose -rate of HTO-exposure was calculated according to the equation (11):

$$\text{rad/hr} = (\text{mCi/ml medium}) \times 12.14 \times 0.8 ,$$

where 12.14 is the conversion factor and 0.8 the water content of the cells.

The duration of irradiation period was 20 hr throughout the experiments and the total accumulated dose was varied by changing the dose-rate.

RESULTS

Effect of Temperature during the Irradiation Period on Cell Killing

The effect of varying the temperature during the irradiation period on the dose-survival relationship is shown in Fig. 1 for γ-irradiation and Fig. 2 for HTO-exposure.

The temperature of 37°C was expected to allow the cells to repair sublethal damage during the 20-hr irradiation period, while the temperature of 4°C was not. This expectation was born out by the facts that the dose-survival curve for 4°C was nearly the same as that obtained after a single acute X-irradiation (8) and that the survival after the irradiation at 37 °C markedly increased in comparison with the survival after the corresponding doses at 4°C (Fig. 1).

The same effect of temperature was observed in the case of HTO-exposure indicating again the existence of repair from sublethal damage during the β-irradiation at 37°C.

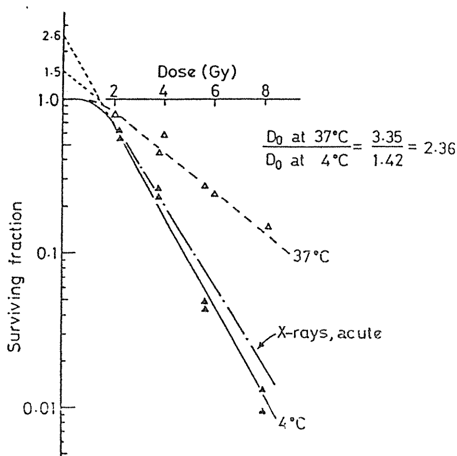


Fig. 1. Dose-survival curves for ^{60}Co γ-rays in 10T1/2 cells. The cells in confluency were kept at either 4 or 37°C during the irradiation period (20 hr). The result for a single acute X-irradiation (0.5 Gy/min) is also indicated for comparison.

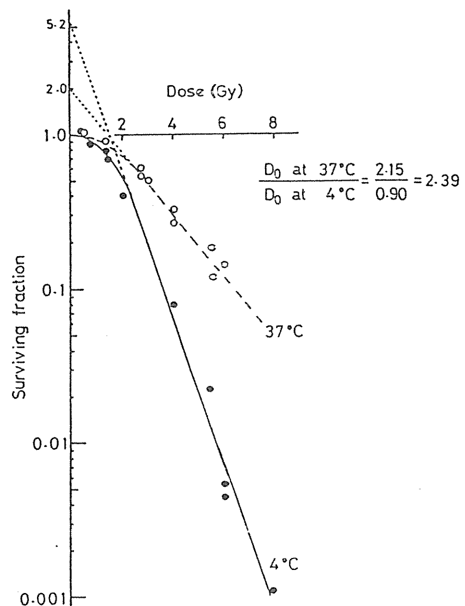


Fig. 2. Dose-survival curves for tritium β-rays in 10T1/2 cells. The cells in confluency were kept at either 4 or 37°C during the irradiation period (20 hr).

Tritium and Gamma Radiation Compared in Cell Killing

Dose-survival relationships were compared between γ-irra-

diation and HTO-exposure in Figs. 3 and 4. Resulting RBE values at D_0 of tritium in comparison with γ-radiation were nearly the same between the two temperature conditions: 1.58 for irradiation at 4°C (Fig. 3) and 1.56 for irradiation at 37°C (Fig. 4).

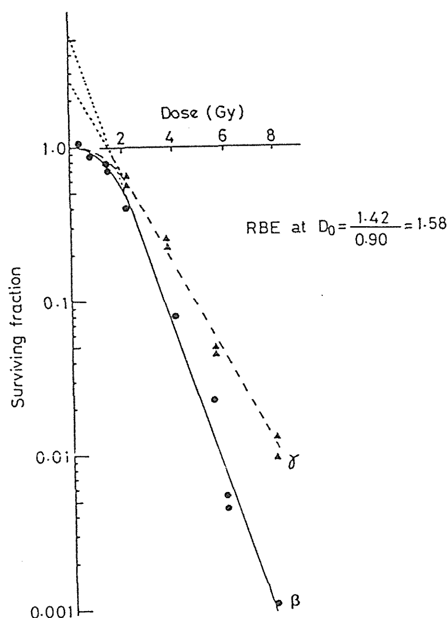


Fig. 3. RBE of tritium β-rays for cell killing in comparison with ^{60}Co γ-rays. The 10T1/2 cells in confluency were kept at 4°C during the irradiation period (20 hr).

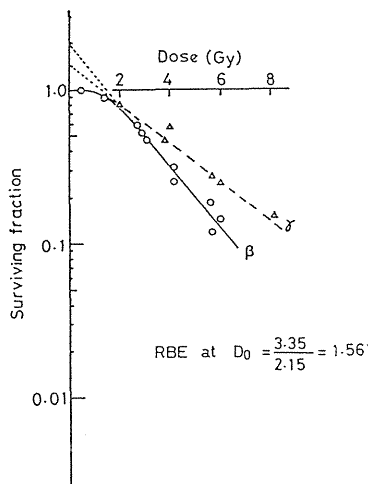


Fig. 4. RBE of tritium β-rays for cell killing in comparison with ^{60}Co γ-rays. The 10T1/2 cells were kept at 37°C during the irradiation period (20 hr).

Effect of Temperature during the Irradaition Period on Transformation Induction

The effect of varying the temperature during the irradiation period on radiation-induced transformation was quite similar to that observed in cell killing. The dose-transformation frequency curve for γ-irradiation at 4°C was again nearly the same as that obtained after a single acute X-irradiation using the same batch of fetal calf serum (Terasima et al., unpublished). The frequency reduced markedly after irradiation at 37°C in

comparison with the frequency after corresponding doses at 4°C (Fig. 5). The same effect of temperature was observed in the case of HTO-exposure (Fig. 6). The results suggest the existence of repair from subtransformational damage during the γ- and β-irradiation at 37°C.

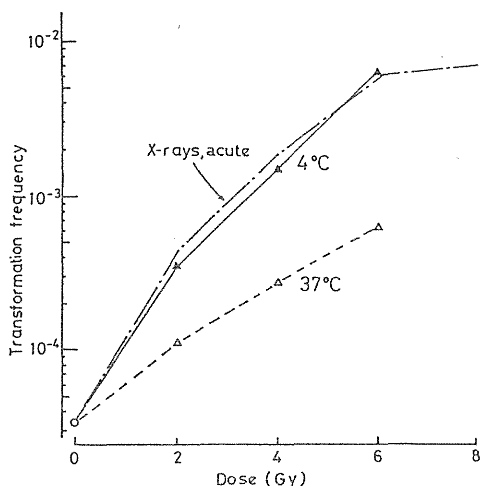


Fig. 5. Dose-transformation induction curves for ^{60}Co γ-rays in 10T1/2 cells. The cells in confluency were kept at either 4 or 37°C during the irradiation period (20 hr). The result for a single acute X-irradiation is also indicated for comparison.

Transformation frequency was expressed as the ratio of the number of transformed foci to the number of surviving colonies in Figs. 5-8.

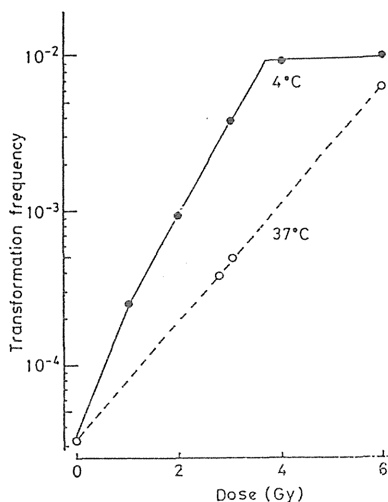


Fig. 6. Dose-transformation induction curves for tritium β-rays in 10T1/2 cells. The cells in confluency were kept at either 4 or 37°C during the irradiation period (20 hr).

Tritium and Gamma Radiation Compared in Transformation Induction

Dose-transformation frequency relationships were compared between γ-irradiation and HTO-exposure in Figs. 7 and 8. As shown in the figures, the RBE value of tritium in comparison with γ-radiation was dose-dependent: The lower the dose, the smaller the RBE value, irrespective of the temperature during the irradiation period. Resulting RBE values of tritium for cell transformation were 1.5-1.8 in the irradiation at 4°C

(Fig. 7) and 1.4-1.8 in the irradaition at 37°C (Fig. 8) within the dose range examined.

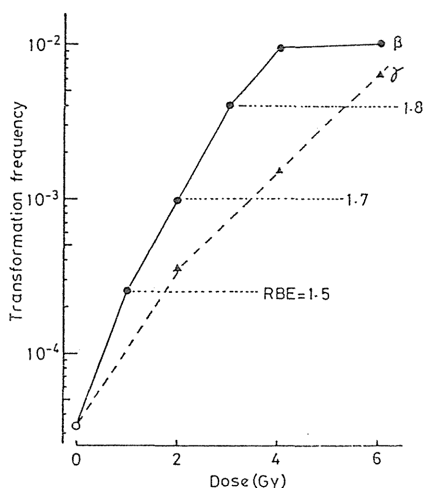


Fig. 7. RBE values of tritium β-rays for transformation induction in comparison with ^{60}Co γ-rays. The 10T1/2 cells in confluency were kept at 4°C during the irradiation period (20 hr).

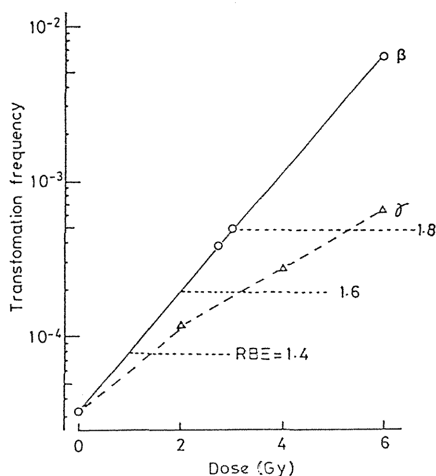


Fig. 8. RBE values of tritium β-rays for transformation induction in comparison with ^{60}Co γ-rays. The 10T1/2 cells in confluency were kept at 37°C during the irradiation period (20 hr).

DISCUSSION

In ICRP publication 9, paragraph 17, the Commission recommended a value of 1.7 as the appropriate quality factor for β^-, β^+, and e^- radiations with maximum energies $\leq$0.03 MeV (12). The value of 1.7 was based on the RBE value obtained for tritium β-rays (5.7 keV) in comparison with ^{60}Co γ-rays in $LD_{50(30)}$ of CF1 mouse (13). Later, however, the Commission decided to issue an amendment to this recommendation and adopted a quality factor of unity for these types of radiations (14).

More recent studies have shown that tritium β-rays may have RBE values greater than 1 for various types of biological effects (7). Thus the value of quality factor for tritium β-rays should be re-examined particularly for its delayed stochastic effects.

The present experiments indicated that the RBE value for cell killing of the 10T1/2 cells in confluency was 1.6 irrespective of the temperature during irradiation. This value corresponds well with those previously reported by many authors for cell killing (7). The RBE values obtained here for *in vitro* transformation of the 10T1/2 cells in confluency were also very close to those obtained for mutation-induction (2-7).

The repair from subtransformational damage in the 10T1/2 cells in the growing phase was evidenced by a dose-fractionation experiment with X-rays (15). In the case of exposure with fission neutrons, the repair was much smaller in extent than that in X-irradiation (15). The result of the present experiment strongly suggested that the transformational damage induced by tritium β-rays, as well as ^{60}Co γ-rays, could be effectively repaired at 37°C during a low-dose-rate irradiation.

If the total dose of X-rays was lower than 1 Gy, the frequency of neoplastic transformation of mammalian cells in culture was reported to increase, rather than decrease, by splitting the dose into fractional doses within several hours (16-18) or by lowering the dose-rate (19). A clear-cut explanation for these paradoxical dose-rate effects is still lacking. We are now attempting to examine the effect of temperature during either β- or γ-irradiation with low dose-rates on the transformation frequency after a total dose lower than 1 Gy.

Low RBE values of tritium β-rays found at low doses were unexpected. This point also needs further study.

REFERENCES

1. ICRP, Publication 26, Pergamon Press, Oxford (1977)
2. H. Tanooka and N. Munakata, Radiat. Res., 73, 581-584 (1978)
3. T. Ito and K. Kobayashi, Radiat. Res., 76, 139-144 (1978)
4. Y. Tazima and K. Onimaru, J. Radiat. Res., 21, 45 (1980)
5. W.L. Russel, R.B. Cumming, E.M. Kelly and E.L. Phipps, IAEA-SM-232/85, 489-497 (1979)
6. S. Okada and N. Nakamura, J. Radiat. Res., 21, 44 (1980)
7. A.M. Ueno, I. Furuno-Fukushi and H. Matsudaira, Radiat. Res., 91, 447-456 (1982)
8. T. Terasima, M. Yasukawa and M. Kimura, Gann, 72, 762-768 (1981)
9. C.A. Reznikoff, J.S. Bertram, D.W. Brankow and C. Heidelberger, Cancer Res., 33, 3239-3249 (1973)
10. H. Otsu, M. Yasukawa and T. Terasima, J. Radiat. Res., 24, 118-130 (1983)
11. W.C. Dewey, R.M. Humphrey and B.A. Jones, Radiat. Res., 24, 214-238 (1965)
12. ICRP, Publication 9, Pergamon Press, Oxford (1966)
13. J.E. Furchner, Radiat. Res., 6, 483-490 (1957)
14. H.J. Dunstler, Health Phys., 17, 389-396 (1969)
15. A. Han and M.M. Elkind, Cancer Res., 39, 123-130 (1979)
16. C. Borek and E.J. Hall, Nature, 252, 499-501 (1974)
17. R.C. Miller, E.C. Hall and H.H. Rossi, Proc. Nat. Acad. Sci. U.S.A., 76, 5755-5758 (1979)
18. J.B. Little, Cancer Res., 39, 1474-1480 (1979)
19. R.J. Hall and R.C. Miller, Radiat. Res., 87, 208-223 (1981)

NIRS-M-52, pp 146 - 156, 1985

Chromosomal Effects of Tritium on Lymphocytes of the Teleost, *Umbra limi*

I.Suyama and H.Etoh*

Divisions of Environmental Health and Biology*
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

Summary

A technique for microculture of lymphocytes from whole blood of the central mudminnow, *U. limi* for chromosome preparations is described.

From within one hour after initiation, blood cultures were exposed at 20 °C to various concentrations (5-100 μCi/ml) of tritiated water for 90 hours for chromosome aberration analysis, and for 144 hours for sister chromatid exchange analysis in the presense of 5-bromodeoxyuridine at 10 μg/ml. To estimate RBE of ^{3}H β-rays relative to ^{137}Cs γ-rays for chromosome aberration induction, blood cultures were irradiated at various dose rates (6.4-23.8 rad/day) of ^{137}Cs γ-rays for 90 hours at 20°C.

Almost all the chromosome aberrations observed were of

chromatid-type. The dose-response relationships for aberration yield were calculated to be Y=0.0610 + 0.0019X for ^{3}H β-rays and Y = 0.0719 + 0.0010X for ^{137}Cs γ-rays using a least-squares method. From slopes of these two regression lines, an RBE value of ^{3}H β-rays relative to ^{137}Cs γ-rays was estimated to be 1.9 for chromosome aberration induction.

The control level of sister chromatid exchanges in *U. limi* lymphocytes is 2.7 SCEs/cell, being lower than that of human lymphocytes. In this study, tritiated water does not significantly increase the incidence of SCEs in lymphocytes of *U. limi*.

Introduction

Since the aquatic environment is becoming more and more polluted with wastes, the availability of fish as a test organism is greatly increasing. In contrast with mammalian systems, however, little progress has been made in using fish systems to study chromosome damage induced by ionizing radiations or other environmental agents. Only a few studies have been reported in which modern cytogenetic techniques were used (1-5).

The lymphocyte culture technique for human chromosome preparations established by Moorhead *et al.*(6) was successfully applied to fish chromosome methodology by Labat *et al.*(7) for the first time. Since then, several investigators have succeeded in culturing fish lymphocytes by slightly modifying this technique (8-12).

All species used, however, are not suitable for cytogenet-

ic study because their complement consists of a large number of small chromosomes and their lymphocytes have a low mitotic activity. Kligerman et al.(1) proposed using the central mudminnow, Umbra limi, as a model animal for the study of chromosome aberrations in fish. U. limi has a low number of large meta- or submetacentric chromosomes (2n=22)(1,13).

In our previous report(14), we described a microculture technique of lymphocytes from whole blood of U. limi. In the present report, effects of tritiated water (HTO) on U. limi lymphocytes are presented with gross chromosome aberrations in M1 cells and sister chromatid exchanges (SCEs) in M2 cells as criteria. In addition, relative biological effectiveness (RBE) of ^{3}H β-rays relative to ^{137}Cs γ-rays is estimated for gross chromosome aberration induction.

Materials and Methods

Sampling of blood

Procurement and maintenance of the central mudminnows, Umbra limi were described elsewhere (14).

Two groups of twelve individuals were established and used alternately. They ranged from 12 to 14 cm in body length. About 0.1 ml of blood at a time was withdrawn from the sinus venosus of the unanesthetized fish into a 1 ml sterilized syringe (Telmo Disposable Syringe, 26Gx1/2) coated with heparin solution. Blood samples were repeatedly taken from the same fish at intervals of 2 weeks without undue harm.

Culture conditions and chromosome preparations

The whole blood from twelve individuals was pooled and

added to the culture medium without any pretreatment. The culture medium was composed of 80 % TC-199 supplemented with 20 % foetal calf serum, streptomycin (100 μg/ml) and penicillin (100 IU/ml). The ratio of whole blood to the culture medium was about one to 50. Before inoculation, phytohemagglutinin (PHA-M, Difco) was added to the medium at the concentration of 0.02 ml/ml as an initiator of mitosis. Colcemid (0.3 μg/ml) was added to the culture flask about 12 h before hypotonic treatment (0.075 M KCl at 30°C) and methanol-acetic acid (3:1) fixation. After air drying, the slides were stained with 5 % Giemsa in Sörensen's phophate buffer (pH 6.8) for chromosome aberration analysis.

For SCE analysis, 5-bromodeoxyuridine (BudR) (10 μg/ml) was added to the blood culture medium aforementioned and colcemid treatment was shortened to 6 hours before harvesting. The slides, which were prepared in the same way as above, were stained by modifying the FPG technique of Perry and Wolff(15) for sister chromatid differentiation (SCD).

Treatment with tritiated water

Within one hour after initiation, blood cultures were exposed at 20°C to various concentrations (5-100 μCi/ml) of HTO for 90 hours for chromosome aberration analysis, and for 144 hours for SCE analysis in the presence of BudR at 10 μg/ml.

Irradiation with ^{137}Cs γ-rays

To estimate RBE of ^{3}H β-rays relative to ^{137}Cs γ-rays for chromosome aberration induction, blood cutures were irradiated at various dose rates (6.4-23.8 rad/day) of ^{137}Cs γ-rays

for 22 hours a day at 20°C. The cultures were harvested 90 hours after the begininng of irradiation, and chromosome aberrations were scored.

Scoring of chromosome aberrations and SCEs

Well-defined metaphase spreads with 20 or more centromeres were selected for both analyses. Ring chromosomes, which are difficult to tell whether they have a centromere and also infrequent, were excluded from chromosome aberration scoring in this study.

Results

1) Treatment with HTO

The frequencies of chromosome aberrations induced in *U. limi* lymphocytes exposed to HTO are shown in Table 1. Almost all the chromosome aberrations observed were of chromatid-type (about 97 %), as was the case with human lymphocytes(16). As for dicentrics, which were the only chromosome-type aberrations scored in this study, one was observed for 10 and 25 μCi/ml group each, and two for 50 and 100 μCi/ml group each. Frequencies of chromosome aberrations were increased with increasing doses. The dose-response relationship was calculated to be $Y = 0.0610 + 0.019X$ using a least-squares method (Fig. 1).

Frequencies of SCE were increased with increasing doses up to 25 μCi/ml, but the level of SCEs at 50 μCi/ml was lower than those at 25 μCi/ml or less (Table 2). At 100 μCi/ml M2 cells were rarely observed.

Table 1. Frequency of Chromosome Aberrations Induced in U. *limi* Lymphocytes Exposed to HTO

HTO Conc. (μCi/ml)	Estimated Dose(rad)	Cells Examined	Aberrant Cells(%)	Chromosome aberrations CG*	CB	ICG	ICB	CE	D	Aberrations /cell ±S.E
0	0	259	13(5.0)	0	5	0	8	0	0	0.050±0.014
5	4.4	239	10(4.2)	2	4	0	4	0	0	0.042±0.013
10	8.7	182	15(8.2)	2	5	0	8	0	1	0.093±0.023
25	21.9	454	47(10.4)	0	19	0	31	1	1	0.119±0.016
50	43.7	345	49(14.2)	1	25	0	26	0	2	0.162±0.022
100	87.4	105	18(17.1)	1	8	0	9	0	2	0.210±0.045

* Abbreviations:
CG:Chromatid gap, CB:Chromatid break, ICG:Isochromatid gap, ICB:Isochromatid break, CE:Chromatid exchange, D:Dicentrics

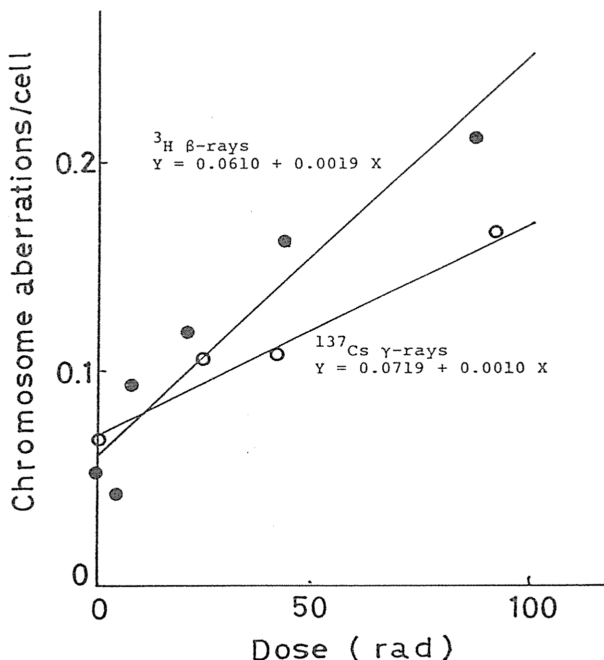


Fig. 1. ^{3}H β-ray and ^{137}Cs γ-ray dose-effect relationships for chromosome aberration induction in U. *limi* lymphocytes

Table 2. Frequency of SCE in U. *limi* Lymphocytes Exposed to HTO for 6 days

HTO Conc. (μCi/ml)	Estimated Dose(rad)	No. of Chromosome	No.of SCE	SCE/cell±S.E.
0	0	30	81	2.70±0.30
5	7.0	23	70	3.04±0.36
10	14.0	30	105	3.50±0.34
25	35.0	15	74	4.93±0.57
50	69.9	18	75	4.17±0.48
100‡				

‡ No M2 cells were observed.

2) Irradiation with ^{137}Cs γ-rays

The results obtained are shown in Table 3. The control level of chromosome aberrations was higher than that of HTO experiments, but the difference between the two was not statistically significant. As was the case with HTO treated lymphocytes, all the chromosome aberrations observed were of chromatid-type. No dicentrics were encountered up to a total dose of 91 rad. The dose-response relationship obtained using a least-squares method i.e., $Y = 0.0719 + 0.0010X$, is shown in Fig. 1 together with that for HTO treated cells.

Table 3. Frequency of Chromosome Aberrations in U. limi Lymphocytes Induced by Exposure to ^{137}Cs γ rays for 90 hours

Dose Rate (rad/day)	Dose (rad)	Cells Examined	Aberrant Cells(%)	Chromosome aberrations						Aberrations /cell ±S.E
				CG*	CB	ICG	ICB	CE	D	
0	0	207	13(6.2)	5	3	1	5	0	0	0.068±0.018
6.4	24.4	178	16(9.0)	1	11	1	6	0	0	0.107±0.024
11.0	42.0	128	11(8.6)	2	4	1	7	0	0	0.109±0.029
23.8	90.9	145	18(12.4)	3	10	0	11	0	0	0.166±0.034

* Abbreviations:
CG:Chromatid gap, CB:Chromatid break, ICG:Isochromatid gap, ICB:Isochromatid break, CE:Chromatid exchange, D:Dicentrics

Discussion

As shown in our preliminary study of cellular kinetics of stimulated U. limi lymphocytes, all the cells arrested at 90 hours after culture initiation at 20 °C were in their first division(14). About 75 % of the cells fixed at 144 hours after initiation were at their second mitosis.

To our knowledge, there have been no reports of chromosome aberration yields in fish lymphocytes induced by HTO. Dose-response relationships of U. limi (present experiments) and human (Hori and Nakai(16)) lymphocytes were shown in Fig. 2. It may be concluded from this figure that U. limi lymphocytes are less sensitive than human lymphocytes to ^{3}H β-rays.

The control levels of chromosome aberrations obtained in the two experiments were not significantly different. An RBE

value of ^{3}H β-rays relative to ^{137}Cs γ-rays was estimated to be 1.9 for chromosome aberration induction under the present experimental conditions. This value is rather high when compared to a value of 1.2 reported for human lymphocytes by Bocian et al.(17) as an RBE of ^{3}H β-rays relative to 180 KV X-rays.

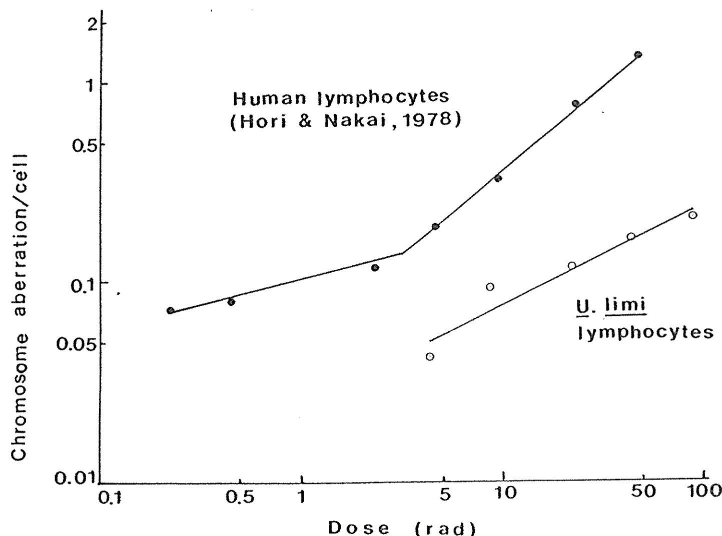


Fig. 2. Comparison between chromosome aberration yields induced in human (16) and U. limi (present experiments) lymphocytes exposed to tritiated water

The control value for SCEs in this study was 2.7/cell. Kligerman and Bloom reported values of 2.4-2.6 SCEs/cell for tissues of U. limi administered i.p. with 500 μg/g BudR and sacrificed 5 to 6 days later(18). A control level of 14.4 SCEs/cell was observed in cultured ovary cells of U. limi (Unpublished data). So, the present value is very close to that of Kligerman and Bloom *in vivo*.

The increase of SCE in HTO treated U. limi lymphocytes was not marked compared to that of gross chromosome aberrations. It is known that SCE is not a sensitive means for detecting damage induced by X-rays in cultured mammalian cells(19). It

can be said that SCE analysis is also not effective for detecting damage in fish lymphocytes irradiated with ^3H β-rays.

As pointed out earlier, the aquatic environment is becoming more and more polluted with various kinds of wastes. Under such circumstances, there is increasing interest in the development of aquatic test systems. In addition to fishes, the mussel, _Mytilus edulis_(20) and the polycheate, _Neanthes arenaceodentata_(21) have already been used as test organisms for environmental monitoring. Along with these _in vivo_ systems, the present _in vitro_ system is expected to become more and more important for detecting effects of water-borne pollutants including radioactive substances. In this connection, studies have been undertaken on chromosomal aberrations following irradiation done before the addition of mitogen with this system.

References

1. A.D.Kligerman, S.E.Bloom and W.M.Howell, _Umbra limi_:A model for the study of chromosome aberrations in fishes. Mutat. Res. 31, 225-233(1975).
2. D.S.Woodhead, Influence of acute irradiation on induction of chromosome aberrations in cultured cells of the fish _Ameca splendens_. In Symposium on Biological Effects of Low-Level Radiation. Vol.1, pp. 67-76. IAEA,Vienna, 1976.
3. S.J.Mong and T.M.Berra, The effects of increasing dosages of X-radiation on the chromosomes of the central mudminnow,

Umbra limi(Kirtland)(Salmoniformes:Umbridae). J.Fish Biol. 14,523-529(1979).

4. R.N.Hooftman, The induction of chromosome aberrations in Notobranchius rachowi (Pisces:Cyprinodontidae) after treatment with ethyl methanesulphonate or benzo[a]pyrene. Mutat. Res. 91,347-352(1981).
5. J.C.M. van der Hoeven, I.M.Bruggeman, G.M.Alink and J.H.Koeman, The killifish Notobranchius rachowi, A new animal in genetic toxicology. Mutat.Res. 97,35-42(1982).
6. P.S.Moorhead,P.C.Nowell,W.J.Mellman,D.M.Battips and D.A.Hungerford, Chromosome preparations of leukocytes cultured from human peripheral blood. Exp.Cell Res. 20,613-616(1960).
7. R. Labat, G.Larrouy and L.Malaspina, Technique de culture des leucocytes de Cyprinus carpio L. C.R.Acad.Sc.Paris 264, 2473-2475(1967).
8. Y.Ojima, S.Hitotsumachi and M.Hayashi, A blood culture method for fish chromosomes. Japan.J.Genet. 45(2),161-162 (1970).
9. J.R.Heckman, F.W.Allendorf and J.E.Wright, Trout leukocytes: Growth in oxygenated cultures. Science 173,246-247(1971).
10. Y.S.Kang and E.H.Park, Leukocyte culture of the eel without autologus serum. Japan.J.Genet. 50(2),159-161 (1975).
11. E.H.Park and H.Grimm, Elevated sister chromatid exchange rate in lymphocytes of the European eel (Anguilla anguilla) with cauliflower Tumor. Cancer Genet. Cytogenet. 5,137-145 (1982).
12. P.C.Balxhall, Lymphocyte culture for chromsome preparation. J. Fish Biol. 22,279-282 (1983).
13. R.J.Beamish, M.J.Merrilees and E.J.Crossman, Karyotypes and

DNA values for members of the suborder Esocoidei (Osteichthyes:Salmoniformes). Chromosoma (Berl.) 34,436-447 (1971).

14. I.Suyama and H.Etoh, X-ray-induced dicentric yields in lymphocytes of the teleost, Umbra limi. Mutat. Res. 107,111-118 (1983).

15. P.Perry and S.Wolff, New Giemsa method for the differential staining of sister chromatids. Nature 251,156-158 (1974).

16. T.Hori and S.Nakai, Unusual dose-response of chromosome aberrations induced in human lymphocytes by very low dose exposure to tritium. Mutat.Res. 50,101-110 (1978).

17. E.Bocian, B.Ziemba-Zak, O.Rosiek and J.Sablinski, Chromosome aberrations in human lymphocytes exposed to tritiated water in vitro. Curr. Top.Radiat.Res.Q., 12,168-181 (1977).

18. A.D.Kligerman and S.E.Bloom, Sister chromatid differentiation and exchanges in adult mudminnows(Umbra limi) after in vivo exposure to 5-bromodeoxyuridine. Chromosoma(Berl.), 56,101-109 (1976).

19. P.Perry and H.J.Evans, Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature, 258,121-125 (1975).

20. F.L.Harrison and I.M.Jones, An in vivo sister-chromatid exchange assay in the larvae of the mussel Mytilus edulis: response to 3 mutagens. Mutat. Res. 105,235-242 (1982).

21. G.G.Pesch, C.E.Pesch and A.R.Malcolm, Neanthes arenaceodentata, a cytogenetic model for marine genetic toxicology. Aqua. Toxi. 1,301-311 (1981).

NIRS-M-52, pp 157 - 172, 1985

Induction of SCE by Tritium in the Mouse Bone Marrow

T.Ikushima[1), R.D.Benz[2)] and A.L.Carsten[2)]

1) Research Reactor Institute
Kyoto University
Kumatori-cho, Sennan-gun, Osaka 590-04, Japan

2) Medical Research Center
Brookhaven National Laboratory
Upton, Long Island, New York 11973, U.S.A.

ABSTRACT

The ability of tritium to induce sister chromatid exchanges (SCEs) and to affect cellular proliferation kinetics has been investigated in the bone marrow cells of mice maintained on water containing 3.0 μCi/ml of tritium in the form of tritiated water (HTO). At intervals ranging from 28 to 261 days after beginning of treatment, the frequency of SCEs and the cell generation time were measured in HTO-ingesting animals and age-matched controls by the bromodeoxyuridine labelling method. The SCE levels in 3.0 μCi/ml HTO-ingesting mice were always higher than those in age-matched control groups. The frequency of induced

SCEs increased linearly with the ingestion time. These results are of particular interest since ionizing radiation is generally not considered to be an efficient inducer of SCEs.

INTRODUCTION

Nuclear fusion could be one of the most promising energy sources in the future. However, this technology may lead to a concentration of tritium in the biosphere several orders of magnitude larger than it is today. It is desirable, therefore, to elucidate the various biological effects of tritium before the first fusion power station is built (1).

Tritium has been considered to be a nuclide with one of the lowest radiotoxicities because it emits a low energy beta particle (Emean = 5.7 KeV) that has a limited range in tissue (6×10^{-4} cm). The ICRP has defined a Q factor of 1.00 for tritium (2). Several papers, however, have reported RBE values for tritium beta particles significantly higher than 1.00 in various biological systems (3,4). Evaluation of both somatic and genetic effects of tritiated water (HTO) is important for the assessment of potential hazard from environmental tritium to man since tritium is present mainly in the environment in the form of water which easily enters into cells or tissues resulting in internal radiation exposure. In addition, tritium from HTO would be incorporated into any hydrogen position in metabolically active macro-molecules including DNA, which is the most sensitive target for various radiobiological effects,

through metabolic pathways (5). The study reported here was undertaken to evaluate the ability of HTO to induce sister chromatid exchanges (SCEs) and to affect cellular proliferation kinetics.

The SCE assay is a sensitive method for measuring the effects of agents that damage chromosomal DNA. Induction of SCEs has been correlated to mutagenesis (6) and neoplastic cell transformation (7).

To mimic the *in vivo* exposure condition, we have examined bone marrow cell chromosomes of mice maintained on water containing 3.0 µCi/ml (100 times the recommended MPC) of tritium in the form of HTO.

MATERIALS AND METHODS

Random-bred male mice of the Hale-Stoner-Brookhaven strain were used for this study. First-litter animals were removed from the mother mice at 4 weeks of age and randomly divided into two groups. One group was maintained on tap water and the other on drinking water containing 3.0 µCi/ml of HTO (New England Nuclear). Animals were kept at a population density no greater than one animal per 97 cm^2 on corn-cob bedding, which was changed weekly. Food and water were provided *ad libitum*. The foods consisted of Purina Laboratory Chow no. 5001(Ralston Purima Company, St Louis, Missouri), a dry pellet food which adds little to the water intake of the animals. The details of tritium metabolism in mice maintained on this regimen have been previously described by Commerford *et al*. (8). All animals,

exposed and control, were maintained in the same room on a 12 hour light : dark cycle at approximately 25 °C, 50 percent relative humidity and with 20 air exchanges per hour.

At selected intervals from 28 to 261 days after first being given HTO, HTO-ingesting and age-matched control mice were placed in restrainers and infused continuously for 24 to 26 hrs through the tail vein with 5-bromo-2'-deoxyuridine (BrdUrd, Sigma) using an infusion device that had a flow rate of approximately 50 mg/Kg body-weight (b.w.) per hour required for SCE visualization (9). Two hours before the termination of the infusion, colchicine (Lilly) was injected intravenously at a concentration of 2 mg/kg b.w. Animals were sacrificed by cervical dislocation, femurs were removed and the bone marrow cells were collected from one femur of each animal. The cells were treated with KCl (75mM), fixed in methanol : acetic acid (3 : 1) and dropped on wet slides. Differential staining was done by a modification of the fluorescence-plus-Giemsa technique (10). Slides were stained in 33258 Hoechst solution (50 µg/ml) for 20 min, rinsed with distilled water, illuminated for 20 min with black light while bathed in phosphate-citrate buffer (pH 7.0), and stained with 4 % Giemsa (Harleco) for 5 min.

The incidence of SCEs was determined in 25 to 50 second-division metaphases having the diploid number of chromosomes (2n = 40) from each animal. Statistically significant differences between groups of animals were determined by Student's _t_ test. To assess cellular proliferation kinetics by the use of harlequine technique,

the proportion of cells that had replicated for one, two or three cycles in a BrdUrd environment was determined in 100 metaphase cells per animal. The average cell cycle time (T) was calculated according to the equation :

$$T(hr) = \text{the duration of infusion (hr)}/(1xA + 2xB + 3xC),$$

where A, B and C are the proportion of the first-, second- and third-division cells, respectively (11).

RESULTS

Fig. 1 shows an example of a harlequine metaphase cell obtained in a femoral bone marrow of a HTO-drinking mouse, having 6 SCEs. The results of the determination of the SCE frequencies on HTO animals are given in Table 1. The SCE levels in bone marrow cells of mice maintained on 3.0 µCi/ml HTO for 28 to 261 days were always higher than those in age-matched control groups. In mice drinking HTO, the number of SCEs per cell ranged from 2.01 to 4.03, while the average frequency varied from 1.71 to 2.81 in unexposed control animals. Statistically significant elevation (1.0 %) of SCE levels above those in controls were seen on 81, 163, 192, 247 and 261 days after the start of continuous ingestion of HTO, and at the 5% level on 72, 86, 227 days. A gradual increase in SCEs relative to controls was observed as a function of the duration of HTO ingestion in mice maintained on HTO, as shown in Fig. 2. Interestingly, the SCE levels of animals maintained on HTO did not rise with age in absolute value but only when compared to control animals in which a gradual decrease

in SCEs was observed as a function of age.

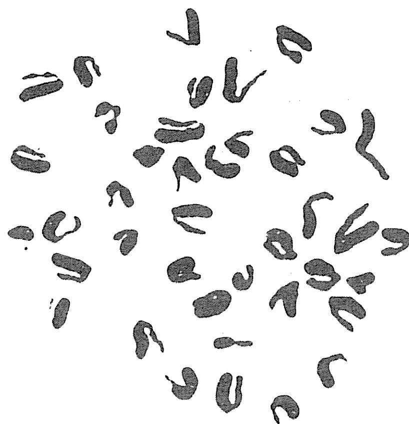


Fig. 1. A harlequine metaphase cell obtained in the bone marrow of a HTO-drinking mouse. 6 SCEs can be counted in the plate.

Table 1. Sister chromatid exchanges in bone marrow cells of BNL/H-S male mice maintained on 3.0 μCi/ml of tritiated water.

Duration of ingestion (day)	Control (HHO)			HTO			Δ(HTO-HHO)	
	No. animals	No. cells	SCE/cell±SEA[a]	No. animals	No. cells	SCE/cell±SEA[a]	SCE/cell±[a]	t-value
28	3	150	2.63 ± .26	3	150	2.95 ± .35	.32 ± .47	.733
72	4	100	2.80 ± .13	4	100	3.25 ± .12	.45 ± .17	2.565*
81	4	100	2.72 ± .19	4	100	4.03 ± .28	1.31 ± .26	3.922**
86	4	100	2.40 ± .27	4	100	3.11 ± .11	.71 ± .28	2.403*
123	4	200	2.53 ± .16	4	200	2.94 ± .23	.41 ± .21	1.496
129	3	150	1.71 ± .05	3	150	2.29 ± .28	.58 ± .12	1.996
136	2	100	1.92 ± .32	4	200	2.01 ± .19	.09 ± .35	.454
163	4	200	2.14 ± .23	3	150	3.30 ± .26	1.16 ± .29	7.227**
186	8	400	2.23 ± .18	6	300	2.35 ± .15	.12 ± .20	.494
192	3	150	2.60 ± .27	3	150	3.77 ± .07	1.17 ± .27	7.221**
227	4	200	2.81 ± .11	4	200	3.60 ± .25	.79 ± .17	2.849*
247	4	200	1.97 ± .17	4	200	3.34 _ .04	1.37 ± .04	7.926**
254	4	200	2.10 ± .20	3	150	3.49 ± .43	1.39 ± .38	1.279
261	3	150	1.95 ± .12	4	200	2.67 ± .14	.72 ± .13	5.542**

[a] Standard error of mean among animals.
* $p < .05$, ** $p < .01$.

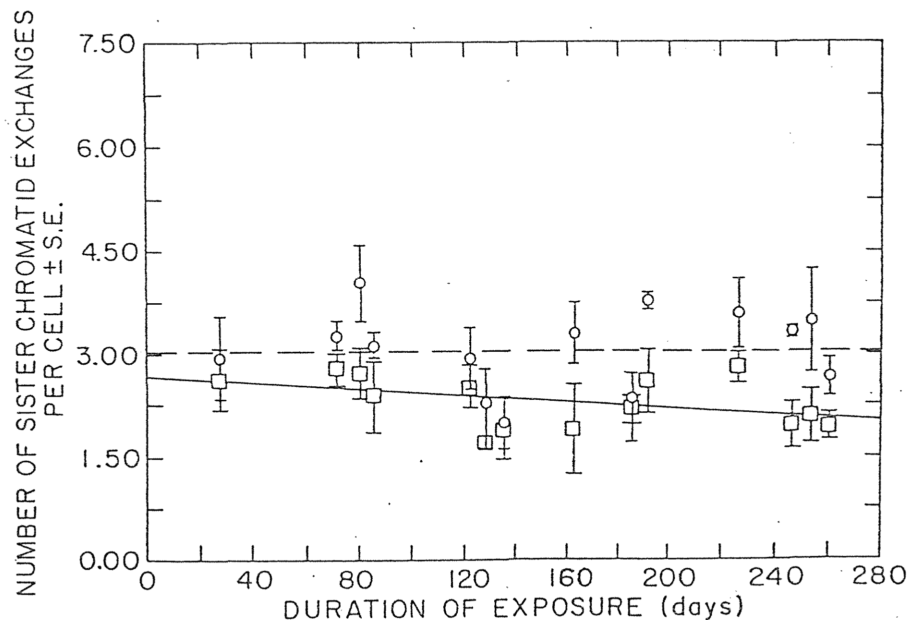


Fig. 2. Frequency of sister chromatid exchanges (SCEs) in bone marrow cells of BNL-H/S male mice maintained on 3.0 μCi/ml of tritiated water (HTO). Squares, control; circles, HTO group.

Using a one-way analysis of variance and covariance, the probability that the mean of all the control data is not different from that of the exposed animals is < 0.0001. The probability that a line regressed through the control data does not have a different slope than one through the exposed data is 0.175.

The frequency distribution of SCEs per cell is shown in Fig. 3, where the HTO ingestion duration is divided into six fractions (I to VI). There is no significant sign of bimodality in the SCE frequency distribution at any age.

The effects of HTO ingestion on cell proliferation is shown in Fig. 4. The continuous intake of 3.0 μCi/ml HTO

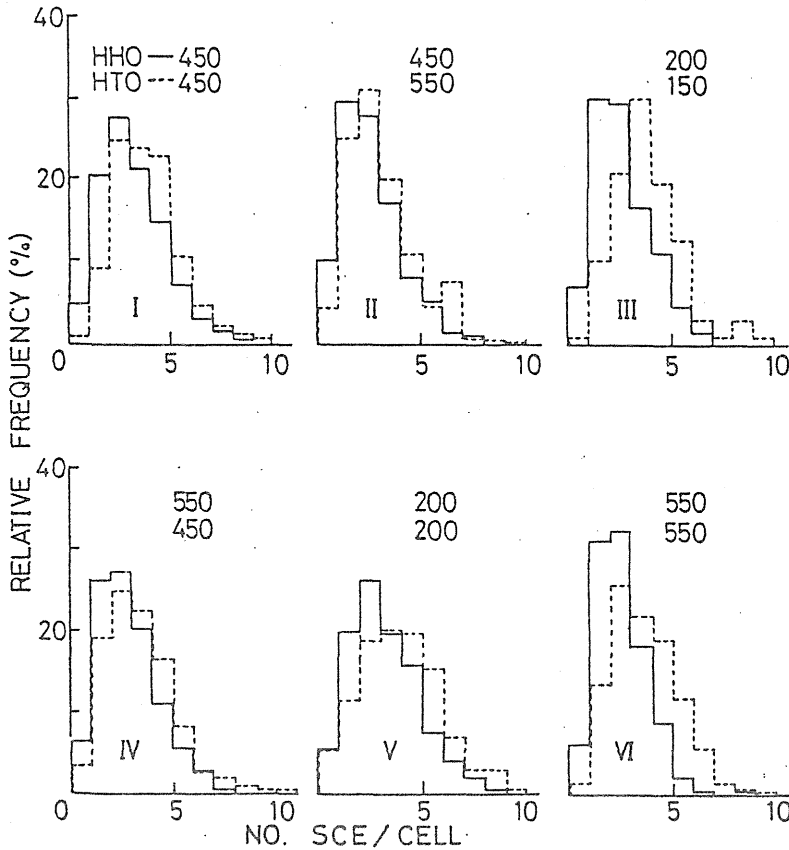


Fig. 3. Relative frequency distribution of SCEs per cell in male mice maintained on 3.0 μCi/ml of tritiated water for: I, 28, 72, 81 and 86 days; II, 123, 129 and 136 days; III, 163 days; IV, 186 and 192 days; V, 227 days; and VI, 247 and 261 days. Numbers are total numbers of cells scored.

caused no significant retardation of proliferation of murine bone marrow cells. The probability that the mean of the control data does not differ from the mean of exposed data is 0.674. The probability that lines regressed through the control and exposed data have slopes that are not different is 0.686.

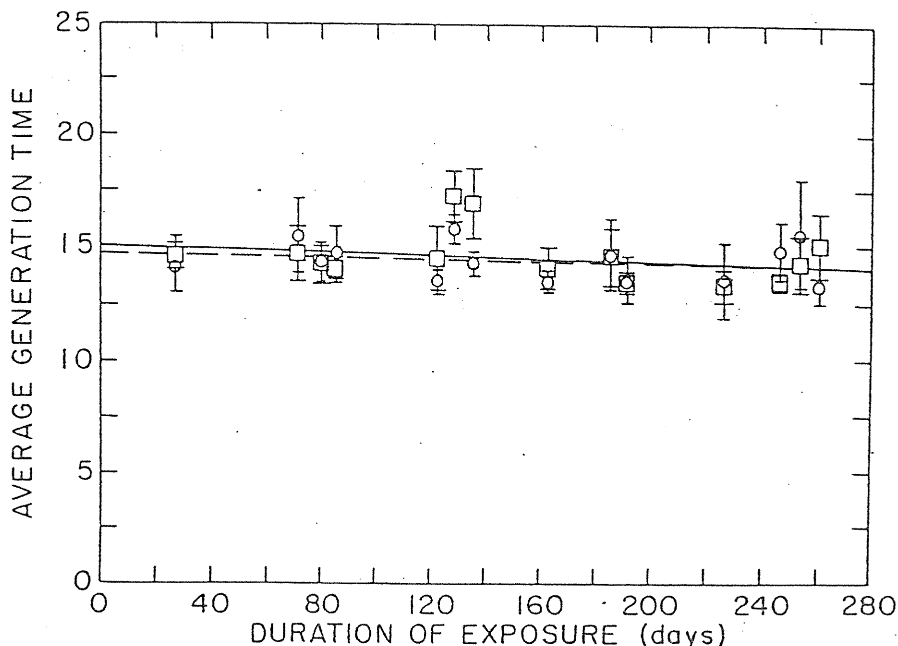


Fig. 4. Average cell generation times in hours of bone marrow cells of BNL/H-S male mice maintained on 3.0 μCi/ml of tritiated water. Squares, control; circles, HTO group.

DISCUSSION

The SCE levels are clearly elevated in femoral bone marrow cells of mice maintained on 3.0 μCi/ml of HTO and the frequency of induced SCEs increases linearly with the ingestion time. HTO groups showed an SCE level as much as 1.7 times higher than that of the age-matched control groups. These results are of particular interest since tritium has not been found to be an efficient inducer of SCEs, as are other external ionizing radiations, as compared to chemical mutagens such as alkylating agents that induce large SCE response at low dose levels (12, 13). Previous studies have indicated that exposure to ionizing radiations

from external sources induce SCEs in cultured mammalian cells (12, 14-22) and murine bone marrow cells (23, 24). Also, the endogenous radiation from tritium incorporated into chromosomes or cell nuclei as ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-lysine inducing SCEs in cultured cells (25-27). It has been shown that both internal tritium and external ionizing radiation do not induce a remarkable increase in the number of SCEs in cells when treated at low doses, and at high doses, this induction appears to reach at limited saturation level. However, none of the studies referred to above examined the effect of tritium ingested as tritiated water or examined the induction of SCEs in animals exposed chronically *in vivo* to ionizing radiation, and so direct comparisons with the results reported in this paper are difficult to make. Nevertheless, we show here that ionizing radiation from ingested HTO is capable of inducing significant numbers of SCEs at very low doses.

Mice which ingest 3.0 µCi/ml of tritium in their drinking water have been measured to reach an equilibrium value of 1.7 µCi/ml of tritium in their bone marrow by 17 days. At equilibium, the radiation dose received by each cell nucleus due to chronic exposure to tritium beta rays has been calculated to be 4.9 mGy/day according to the equation: 1.0 µCi/ml = 2.9 mGy/day (8). On this basis a dose of only 0.98 Gy would accumulate over 200 days of ingestion of 3.0 µCi/ml of HTO. The results presented here indicate that such a low dose of low energy (E_{max} = 0.018 MeV) beta rays can result in the induction of SCEs in murine

bone marrow cells. It may be that chronic exposure to HTO causes SCE-producing lesions in chromosomal DNA that somehow persist even in the DNA of rapidly dividing cells, or SCEs may result from lesions in the DNA of cells that were non-cycling until soon before the time of SCE analysis, or there may be some, unknown, secondary effects of HTO on the cell renewal system of bone marrow.

Several reports have appeared describing somatic and genetic effects of long term HTO ingestion in animals. In mice continuously maintained for 330 days on 3.0 µCi/ml of HTO in the same regimen as used in this study, the frequency of chromosomal aberrations in the regenerating liver increased significantly over the control values (28). When the accumulated dose reached 0.77 Gy after about 23 weeks of HTO ingestion, the number of bone marrow haemopoietic stem cells decreased significantly (29). Dominant lethal mutations in mice maintained on 3.0 µCi/ml of HTO showed a slight but significant increase in mutation rate (30). However, there is no _a priori_ reason to assume that a common molecular mechanism exists for the induction of SCEs, chromosomal aberrations, dominant lethal mutations and haematological effects (31). Elevated SCE levels in mice maintained on HTO may, also, not be mechanically related to the effects of tritiated water on cellular proliferation, since no difference in average cell generation time was seen between HTO and control groups.

Our experiments, however, suggest that the SCE assay is useful for monitoring cytogenotoxicity of internal exposure to relatively low level chronic tritium radiation.

ACKNOWLEDGEMENTS

This work was supported by the fund of US-Japan Fusion Cooperation Program to T.I. and the U.S. Department of Energy under Contract No. DE-AC02-76CH00016.

A part of this study has been appeared in the paper: IKUSHIMA, T., R. D. BENZ and A. L. CARSTEN, Sister chromatid exchanges in bone marrow cells of mice maintained on tritiated water. Int. J. Radiat. Biol. 45, 251-256 (1984).

REFERENCES

1. FEINENDEGEN, L. E., E. P. CRONKITE and V. P. BOND, Radiation problems in fusion energy production. Radiat. Environ. Biophys. 18, 157-183 (1980).

2. DENSTER, H. J., "News and Comment", Health Phus. 17, 389-396 (1969).

3. ELLET, W. H. and L. A. BRABY, The microdosimetry of 250 kVp and 65 kVp X-rays, 60-Co gamma rays, and tritium beta particles.Radiat. Res. 51, 229-243 (1972).

4. UJENO, Y., Relative biological effectiveness (RBE) of tritium beta rays in relation to dose rate. Health Phys. 45, 789-791 (1983).

5. CARSTEN, A.L., S. L. COMMERFORD and E. P. CRONKITE, The genetic and late somatic effects of chronic tritium ingestion in mice. Curr. Topics Radiat. Res. Q. 12, 212-224 (1977).

6. CARRANO, A. V., L. H. THOMPSON, P. A. LINDL and J. L. MINKLER, Sister chromatid exchange as an indicator

of mutagenesis. Nature 271, 551-553 (1978).

7. POPESCU, N. C., S. C. AMSBAUGH and J. A. DIPAOLO, Relationship of carcinogen-induced sister chromatid exchange and neoplastic cell transformation. Int. J. Cancer 28, 71-77 (1981).

8. COMMERFORD, S. L., A. L. CARSTEN and E. P. CRONKITE, The distribution of tritium in the glycogen, hemoglobin, and chromatin in mice receiving tritium in their drinking water. Radiat. Res. 72, 333-342 (1977).

9. TICE, R., J. CHAILLET and E. L. SCHNEIDER, Demonstration of spontaneous sister chromatid exchanges in vivo. Exp. Cell Res. 102, 426-428 (1976).

10. PERRY, P. and S. WOLFF, New Giemsa method for the differential staining of sister chromatids. Nature 251, 156-158 (1974).

11. IVETT, J. and R. TICE, Average generation time: A new method of analysis and quantitation of cellular proliferation kinetics. Environ. Mut. 4, 358 (1982).

12. PERRY, P. and H. J. EVANS, Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258, 121-125 (1975).

13. SOLOMAN, E. and M. BOBROW, Sister chromatid exchange — A sensitive assay of agents damaging human chromosomes. Mutat. Res. 30, 273-278 (1975).

14. MARIN, G. and D. M. PRESCOTT, The frequency of sister chromatid exchanges following exposure to varying doses of ^{3}H-thymidine or X-rays. J. Cell Biol. 21, 159-167 (1964).

15. WOLFF, S., J. BODYCOTE and R. B. PAINTER, Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation of different stages of the cell cycle: The necessity for cells to pass through S. Mutat. Res. 25, 73-81 (1974).

16. ABRAMOVSKY, I., G. VORSANGER and K. HIRSCHHORN, Sister chromatid exchange induced by X-ray of human lymphocytes and the effect of L-cysteine. Mutat. Res. 50, 93-100 (1978).

17. NAKATSUGAWA, S., K. ISHIZAKI and T. SUGAHARA, The reduction in frequency of X-ray-induced sister chromatid exchanges in cultured mammalian cells during post-irradiation incubation in Hank's balanced salt solution. Int. J. Radiat. Biol. 34, 489-492 (1976).

18. NAGASAWA, H. and J. B. LITTLE, Effect of tumor promoters, protease inhibitors, and repair processes on X-ray-induced sister chromatid exchanges in mouse cells. Proc. Natl. Acad. Sci. USA 76, 1943-1947 (1979).

19. LIVINGSTON, G. K. and L. A. DETHLEFSEN, Effects of hyperthermia and X irradiation on sister chromatid exchange (SCE) frequency in Chinese hamster ovary (CHO) cells. Radiat. Res. 77, 512-520 (1979).

20. MORGAN, W. F. and P. E. CROSSEN, X-irradiation and sister chromatid exchange in cultured human lymphocytes. Environ. Mut. 2, 149-155 (1980).

21. RENAULT, G., A. GENTIL and I. CHOUROULINKOV, Kinetics of induction of sister-chromatid exchanges by X-rays through two cell cycles. Mutat. Res. 94, 359-368 (1982).

22. IKUSHIMA, T., Radiation-induced sister chromatid exchanges. Jap. J. Genet. 58, 648 (1983).

23. NAKANISHI, Y. and E. L. SCHNEIDER, In vivo sister chromatid exchanges: A sensitive measure of DNA damage. Mutat. Res. 60, 329-337 (1975).

24. MORALES-RAMÍREZ, P., I. VALLARINO-KELLY and R. RODRIGUEZ-REYES, Effect of BrdUrd and low doses of gamma radiation on sister chromatid exchange, chromosome breaks, and mitotic delay in mouse bone marrow cella in vivo. Environ. Mut. 5, 589-602 (1983).

25. GIBSON, D. A. and D. M. PRESCOTT, Induction of sister chromatid exchanges in chromosomes of rat kangaroo cells by tritium incorporated into DNA. Exp. Cell Res. 74, 397-402 (1972).

26. CROSSEN, P. E. and W. F. MORGAN, The effects of β-radiation on sister-chromatid exchanges in cultured human lymphocytes. Mutat. Res. 62, 125-129 (1979).

27. IKUSHIMA, T., Mechanism of sister chromatid exchange formation — Intrachromosomal distribution. J. Radiat. Res. 21, 47 (1980).

28. BROOKS, A. L., A. L. CARSTEN, D. K. MEAD and J. C. RETHERFORD, The effect of continuous intake of tritiated water (HTO) on the liver chromosomes of mice. Radiat. Res. 68, 480-489 (1976).

29. CARSTEN, A. L. and E. P. CRONKITE, Comparison of late effects of single X-ray exposure, chronic tritiated water ingestion, and chronic Cesium-137 gamma exposure in mice. IAEA-SM-237/45, I.A.E.A., Vienna, pp. 269-276(1979).

30. CARSTEN, A. L. and S. L. COMMERFORD, Dominant lethal mutations in mice resulting from chronic tritiated water (HTO) ingestion. Radiat. Res. 66, 609-614 (1976).

31. IKUSHIMA, T., Role of sister chromatid exchanges in chromatid aberration formation. Nature 268, 235-236 (1977).

NIRS-M-52, pp 173 - 181, 1985

Development of the Monitoring System for Human Expsoure to Tritium. Chromosome Aberrations in Human Lymphocytes Expsoed at G_0 to HTO

K.Morimoto

Department of Public Health
Faculty of Medicine
University of Tokyo
7-3-1 Hongo, Tokyo 113, Japan

ABSTRACT

Human lymphocytes at G_0 were exposed *in vitro* to HTO (1) to obtain the dose-response relationship between exposure and the dicentric and ring (D+R) frequency, (2) to see the dose-rate effect on the chromosome aberration frequency, and (3) to investigate the enhancing effect of the combined treatment with metabolic inhibitors on the HTO-induced chromosome aberrations. The dose-response curve obtained was shown to be fitted by a power model:

$$Y = 0.205\ X^{1.29},$$

or a linear quadratic model:

$$Y = 1.5 \cdot 10^{-3}\ X + 1.9 \cdot 10^{-6}\ X^2,$$

where X is HTO exposure in rad and Y is the D+R frequency. When

lymphocytes were exposed to HTO at 3 different dose rates, it was found that exposure at 0.042 Gy/h produced a 30% smaller number of D+R chromosomes than exposure at 0.5 Gy/h. In cultures exposed to 2-Gy HTO β-rays along with various metabolic inhibitors such as cytosine arabinoside (ara-C), 3-aminobenzamide (3-AB), hydroxyurea (HU), fluorodeoxyuridine (FUdR), caffeine, and cycloheximide (CHX), a remarkably enhanced ratio of D+R frequencies was observed when cells were combinedly exposed to ara-C, varying from 2 to 5 among lymphocytes from different blood donors. Caffeine, FUdR or 3-AB also enhanced HTO-induced chromosome aberrations by 20-50% whereas HU and CHX appear not to affect the HTO-induced D+R frequency.

INTRODUCTION

The concentration of tritium in our environment, once increased due to H-bomb weapon tests, has been decreasing since mid 1960s. In the future, however, reprocessing plants of the used nuclear reactor fuel and nuclear fusion reactors will release quantities of tritium into our environment. It is thus necessary to develop a human monitoring system to protect members of public against exposure to this radionuclide.

It has widely been shown that lymphocytes from persons exposed to radiation, or those irradiated in vitro at G_0 show a dose-dependent increase in chromosomal aberrations when cultured for 2 - 3 days (see refs. 1 and 2). A lot of experiments have been carried out to obtain precise dose-response relationships for various radiation qualities such as X-rays, gamma-rays, alpha-particles and neutrons, and the dose-response curve has been shown to be used for estimating the absorbed dose by the persons accidentally exposed to radiation (3).

There is however little information available for the dose-response relationship with β-rays emitted from tritiated water (HTO).

As the first step to develop such a cytogenetic monitoring system for human exposure to HTO, experiments have been done on human lymphocytes *in vitro* (1) to obtain the dose-response relationship between HTO exposure and the dicentric and ring (D+R) frequency, (2) to see the dose-rate effect on the induced aberration frequency, and (3) to investigate the combined effect of various metabolic inhibitors.

MATERIALS AND METHODS

Lymphocyte culture and slide preparation. Heparinized peripheral blood samples were drawn from healthy adults. Whole blood (0.3 ml) was added to 4.7 ml of RPMI medium 1640 (GIBCO) containing 15% fetal bovine serum (GIBCO) and 3% phytohemagglutinin (PHA)-M (Difco). The cultures were incubated at 37°C for 52 h in complete darkness. At 24th-h after the initiation of the culture, colcemid (final concentration, 2×10^{-7} M) was added to the each culture, and air-dried chromosome preparations were made as previously described (4). With this culture method exclusively first division cells were sampled.

HTO-treatment. For treatment with HTO, whole blood was suspended in serum-free culture medium containing HTO at the appropriate concentration at 37 °C, giving doses ranging from 0.25 Gy to 5.6 Gy at various dose-rates before PHA-stimulation. The HTO β-ray dose was evaluated by measuring radioactivity of the treating medium by a liquid scintillation counter. To terminate the treatment, cells were centrifuged at 1500 rpm for 5 min and washed five times with pre-warmed phsphate-buffered saline (PBS). The cells were then

resuspended in complete culture medium containing 3% PHA to initiate the culture.

γ-irradiation. For γ-irradiation, whole blood in serum-free culture medium was exposed to ^{137}Cs-γ-rays at 37°C at a dose-rate 0.5 Gy/h, except otherwise stated. The cells were washed three to five times immediately after the irradiation, and pre-warmed complete medium containing 3% PHA was added to initiate the culture.

Chemical treatment. For the cultures treated with metabolic inhibitors, caffeine, cytosine arabinoside (ara-C), 5-fluoro-2'-deoxyuridine (FUdR), cycloheximide (CHX), hydroxyurea (HU) were first dissolved in distilled water, 3-aminobenzamide (3-AB) in dimethylsulphoxide, and after necessary dilutions with PBS aliquots of these freshly made solutions were added to each culture to give appropriate final concentrations during exposure to HTO β-rays or γ-rays. The cells were washed three or five times immediately after the irradiation, and pre-warmed complete medium containing 3% PHA was added to initiate the culture.

Scoring. One hundred and fifty cells were scored per point per person blindly on coded slides.

RESULTS AND DISCUSSION

(1) Optimal culture method

Although 2-day cultures of PHA-stimulated human lymphocytes have been used to get dose-response curves between radiation exposure and induced chromosomal abrrations, I previously showed that even

2-day cultures have a considerable percentage of 2nd-division cells (Fig. 1; 5-7). The frequency of dicentric and ring chromosomes decreased by 50% through each division (8-11). To get correct dose-response relationships, therefore, it is necessary to score the chromosomal aberrations exclusively on 1st-division cells. In the following experiments, Colcemid was added at the 24th-h after the PHA-stimulation to collect exclusively 1st-division cells in the resultant cultures. I have also noted that the lymphocyte cultures added colcemid at 24th-h have higher frequencies of dicentric and ring chromosomes than those added at the beginning of the cultures when exposed to γ-rays (data not shown).

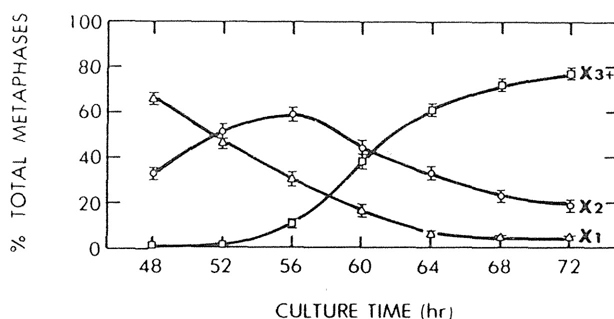


Fig. 1 Percentages of 1st (X_1), 2nd (X_2), and 3rd or subsequent (X_{3+}) division cells in cultures fixed at different times after stimulation of cultures.

(2) <u>Chromosome aberrations (dicentric and ring chromosomes) in human lymphocytes exposed to HTO in G_0</u>

The frequency of dicentric and ring chromosomes, which are a good indicator of radiation exposure, has been used as an indicator of radiation-induced chromosomal aberrations throughout the following experiments. When cells exposed to various concentrations of HTO were examined, the results obtained could be fitted to a power model:

$$Y = 0.205\ X^{1.29},$$

or to a linear quadratic model:

$$Y = 1.5 \cdot 10^{-3} X + 1.9 \cdot 10^{-6} X^2,$$

where X is HTO exposure in rad on the assumption that treatment with 10 mCi/ml HTO for 1 h results in an absorbed dose of 1 Gy, and Y is the dicentric and ring frequency per cell (Fig. 2).

The dose-response curves obtained with the other radiations are also examined (data not shown).

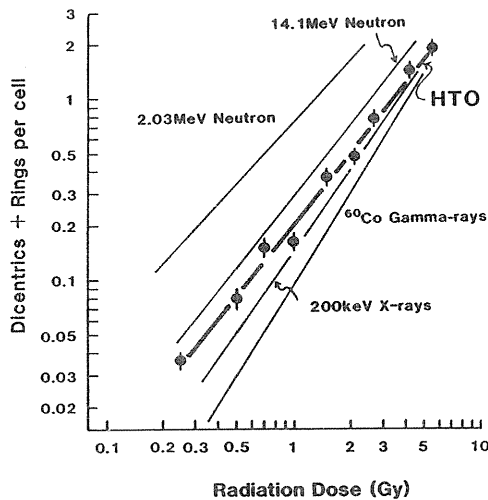


Fig. 2 Dose-response relationship between HTO β-ray dose and dicentric and ring frequency.

(3) Dose-rate effect

Human lymphocytes in G_0 were exposed to HTO β-rays at 3 different dose rates. The data show that exposure at a dose-rate of

Table 1

Dose-rate Effect on the HTO-induced Dicentrics and Rings

	Dose-rate (Gy/h)		
	0.500	0.083	0.042
D + R/c[a]	0.18 ± 0.02[b]	0.20 ± 0.03	0.12 ± 0.02
Ratio	1.00	1.13	0.67

[a] Dicentrics plus rings per cell.
[b] Mean ± S.E. of 300 cells.

0.042 Gy/h produced about 30% smaller numbers of dicentrics and rings compared with those produced at 0.500 Gy/h (Table 1).

(4) Effects of metabolic inhibitors on the HTO-induced choromosome aberrations

Human lymphocytes were treated with HTO along with various metabolic inhibitors (Caffeine; FUdR; CHX; 3-AB; HU; and Ara-C) for 4 h immediately before PHA-stimulation of culture. A remarkably enhanced ratio of dicentric and ring frequencies was observed in cells treated with ara-C; ara-C treatment resulted in 4 times higher frequency of dicentrics and rings compared to that in cells exposed to

Table 2

Effects of Metabolic Inhibitors on the Frequency of the Chromosome Aberrations Induced by HTO β-ray or γ-ray Exposure.

Inhibitor	Enhancement Ratio	
	HTO	γ-ray
Caffeine 500 μg/ml	1.37	1.67
FUdR 10 μg/ml	1.54	1.33
CHX 10 μg/ml	1.01	1.08
3AB 10 mM	1.18	1.25
HU 5 mM	1.00	1.17
Ara-C 50 μM	4.20	1.95

Enhancement ratio:

$$\frac{(D + R)_{HTO + ara\text{-}C}}{(D + R)_{HTO} + (D + R)_{ara\text{-}C}}$$

HTO only (Table 2). Caffeine and FUdR also enhanced HTO-induced chromosome aberrations by 30-50%. 3-Aminobenzamide, a poly(ADP-ribose) polymerase inhibitor, produced about 20% higher frequencies of dicentric and ring chromosomes when compared with those produced by HTO exposure only. The other inhibitors, CHX, a protein synthesis inhibitor, and HU, a DNA synthesis inhibitor, produced almost no effects on the chromosome aberrations induced by HTO β-rays.

When enhancement ratios were calculated for lymphocytes from 10 blood donors, it was shown that they varied from 2 to 5. It also appeared that lymphocytes that had relatively higher frequencies of dicentric and ring chromosomes had conversely lower frequencies of those aberrations when exposed to HTO along with ara-C.

ACKNOWLEDGEMENTS

I thank K. Miura and M. Mizuno for excellent assistance. Work supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan, and from the Nissan Science Foundation.

REFERENCES

1. H.J. EVANS and D.C. LLOYD (Eds.), Mutagen-induced Chromosome Damage in Man. Yale Univ. Press, New Haven, 1978.
2. H.J. EVANS, W.M. COURT BROWN and A.S. McLEAN (Eds.), Human Radiation Cytogenetics. North Holland, Amsterdam, 1967.
3. T. ISHIHARA, S. KONDO, K. HIRASHIMA, T. KUMATORI, H. SUGIYAMA and KURISU, Chromosome aberrations in persons accidentally exposed to ^{192}Ir gamma-rays. J. Rad. Res. 14, 328 (1973).
4. K. MORIMOTO, Induction of sister chromatid exchanges and cell

division delays in human lymphocytes by microsomal activation of benzene. Cancer Res. 43, 1330-1334 (1983).

5. K. MORIMOTO, M. SATO and A. KOIZUMI, Proliferative kinetics of human lymphocytesin culture measured by autoradiography and sister chromatid differential staining. Exp. Cell Res. 145, 349-356 (1983).
6. K. MORIMOTO and S. WOLFF, Cell cycle kinetics in human lymphocyte cultures. Nature 288, 604-606 (1980).
7. K. MORIMOTO and S. WOLFF, Increase of sister chromatid exchanges and perturbations of cell division kinetics in human lymphocytes by benzene metabolites. Cancer Res. 40, 1189-1193 (1980).
8. A.V. CARRANO and J.A. HEDDLE, The fate of chromosome aberrations. J. Theoret. Biol. 38, 289-304 (1973).
9. K. MIURA, K. MORIMOTO and A. KOIZUMI, Proliferative kinetics and mitomycin C-induced chromosome damage in Fanconi's anemia lymphocytes. Hum. Genet. 63, 19-23 (1983).
10. D. SCOTT and C.Y. LYONS, Homogeneous sensitivity of human peripheral blood lymphocytes to radiation-induced chromosome damage. Nature 278, 756-758 (1979).
11. K. MORIMOTO, T. KANEKO, K. IIJIMA and A. KOIZUMI, Proliferative kinetics and chromosome damage in trisomy 21 lymphocyte cultures exposed to γ-rays and bleomycin. Cancer Res. 44, 1499-1504 (1984).

NIRS-M-52, pp 182 - 192, 1985

UV Sensitivity of the Mouse Sperm and Zygotes Fertilized *In Vitro*

T.Yamada[1], Y.Matsuda[2], H.Ohyama[3] and A.Ohkawa[1]

Divisions of Biology[1], Genetics[2] and Radiation Health[3]
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

Since the early work of Russell and Montgomery (1965), it is well known that preimplantation mouse embryos have the highest radiosensitivity and then the radiosensitivity varies depending on the developmental stage as well as the cell cycle. Such sensitivity changes in the zygote stage were not thus far examined because of technical difficulties. We established a complete culture system from the eggs fertilized *in vitro* to the expanded blastocyst with high efficiency by using ICR and $BC3F_1$ mice (Radait. Res., 92, 359, 1982). Using this system, we examined radiosensitivity changes of mouse zygote stage (fertilization to the 1st cleavage) and found that the early pronuclear zygotes had the highest sensitivity to radiation including HTO

This work was supported by a special grant for Tritium Research from the Science and Technology Agency and in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan

β-rays and sperm was rather resistant (Proc. 1st Workshop on Tritium Radiobiol. & Health Pysics, NIRS-M-41, 68, 1982, J. Radiat. Res., 23, 450, 1982). In the present study, we expanded the same series of experiments to UV sensitivity including sperm immediately before insemination which can be examined exclusively by the in vitro fertilization method. In contrast to radiosensitivity, UV-sensitivity of sperm and the pronuclear zygotes fell in almost the same range(LD-50, half-lethal fluence: about 2.6 J/m^2).

INTRODUCTION

Early mammalian embryos at preimplantation stages of development can now be cultured successfully in the chemically defined media. Consequently, it is now possible to assess embryotoxic potential of ionizing radiation and chemical agents without any influence of the maternal reproductive tract. We established a complete culture system for cultivating mouse eggs fertilized in vitro to the expanded blastocyst stage with high efficiency (1, 2). The in vitro fertilization and the subsequent culture techniques enable us to irradiate mature germ cells immediately before fertilization or zygotes at a precise time after fertilization.

Using this system, we examined the sensitivity of cultured mouse embryos to HTO β particles. The embryos at earlier developmental stages were found to be more sensitive, and the pronuclear zygotes had the highest sensitivity. RBE for HTO β radiation relative to ^{60}Co γ-rays was determined to be 1 to 1.7 (1). Further, we measured the radiosensitivity of mouse zygotes fertilized in vitro to X-rays as a function of time from fertilization to the first cleavage. The dose of X-rays (LD-50)

required to prevent development of 50 % of the zygotes to the blastocyst stage *in vitro* varied markedly depending on the time of irradiation from about 40 to 400 R. The zygotes in early pronuclear stage (4-6 hr after insemination) had the highest radiosensitivity (2).

In the present study, we extended the same series of experiments to UV sensitivity including sperm immediately before insemination which can be examined exclusively by the *in vitro* fertilization method.

MATERIALS AND METHODS

Animals

Female BC3F$_1$ (C57BL/C3H) and male ICR mice were used throughout the experiments. All animals were maintained on a light schedule of 07:00 to 19:00 and were given food and water *ad libitum*.

In vitro fertilization and embryo culture

Fertilization of the mouse eggs *in vitro* and subsequent culture of the zygotes were carried out as described previously (1). Briefly, ten to 15 week old females were hormonally superovulated with 8 IU of pregnant mares' serum gonadotrophin (PMSG) followed 48 hr later by 8 IU of human chorionic gonadotrophin (hCG). Fifteen hours after hCG administration the mice were sacrificed and tubal eggs were obtained. Sperm had been obtained from cauda epididymis of 3 to 4 month old ICR male mouse and capacitated by incubation with fertilization medium (1) at 37°C for 1 to 1.5 hr. Sperm suspension was added to the medium containing eggs to give a final sperm concentration of 50 to 150

sperms/μl. At 3 to 5 hr after insemination, the fertilized eggs, which were confirmed by the extrusion of the 2nd polar bodies, were washed twice and transfered to the cultured medium (1). Efficiency of blastocyst formation of the normal zygotes was more than 95 %.

UV-irradiation of the in vitro fertilized zygotes at various times during zygote stage

The zygotes were collected, washed twice to remove surrounding cumulus and pooled in the culture medium without bovine serum albumin (BSA). Aliquots of 20 to 30 zygotes were put into separate wells of spot dishes (15 mm diameter) and UV-irradiated from above with 0 to 12 J/m^2. The wells contained 0.2 ml of the BSA-depleted culture medium and the maximum depth of the medium was 1.5 mm. The UV absorption of the medium at 254 nm is negligible. For UV-irradiation sperm collected from cauda epididymis was suspended with the medium free of BSA having the maximum depth less than 1 mm, and irradiated. Immediately after the irradiation, sperm or the zygotes were transferred to the normal medium containing BSA. The UV source for irradiation was a HITACHI GL 15 germicidal lamp, and its 254 nm-light intensity at the position of the spot dish for sperm or zygote irradiation was measured by an UVX-Radiometer equipped with UVX-25 sensor (Ultra Violet Product, Inc., CA. USA).

Estimation of half lethal UV fluence (LD-50)

The killing action of UV on the zygotes in various phases of the cell cycle was investigated using the end-point of blastocyst formation in vitro and quantitated by that fluence causing 50 % lethality, LD-50. The action of UV on sperm immediately before

insemination was investigated also using the end-point of blastocyst formation after fertilization with normal eggs. They were analyzed by probit tranformation on a computor to estimate the LD-50 and its 95 % confidence limits and standard error for each series of experiments.

RESULTS

UV-Sensitivity variation within the zygote stage

The half lethal UV fluence (LD-50) values were determined for sperm and zygotes at various stages (4 to 12 hr after insemination) from insemination to the first cleavage as shown in Fig. 1. In Fig. 2, the LD-50 values of various zygote groups were plotted against the time of irradiation after insemination. The UV sensitivity varied markedly depending on the times of irradiation.

The zygotes fertilized with UV-irradiated sperm have relatively lower LD-50 value, in contrast to the zygotes fertilized with X-irradiated sperm, which showed higher LD-50 value (2). The UV sensitivity reached maximum (LD-50 is 1.4 J/m^2) 4 hr after insemination which corresponds to the stage at the beginning of pronuclear formation. Thereafter, the sensitivity decreased progressively through the completion of the pronuclear formation (12 hr after insemination) to minimum at 12 hr after insemination (LD-50; 10.7 J/m^2). The sensitivity increased again with progression of the development to the first cleavage. These results indicate that the zygotes were the most sensitive to UV at the beginning of pronuclear formation and the most resistant at the end of the pronuclear stage. This pattern is exactly similar to the pattern for X-irradiated zygotes (2), and is in contrast to the pattern for UV-irradiated somatic cells (3).

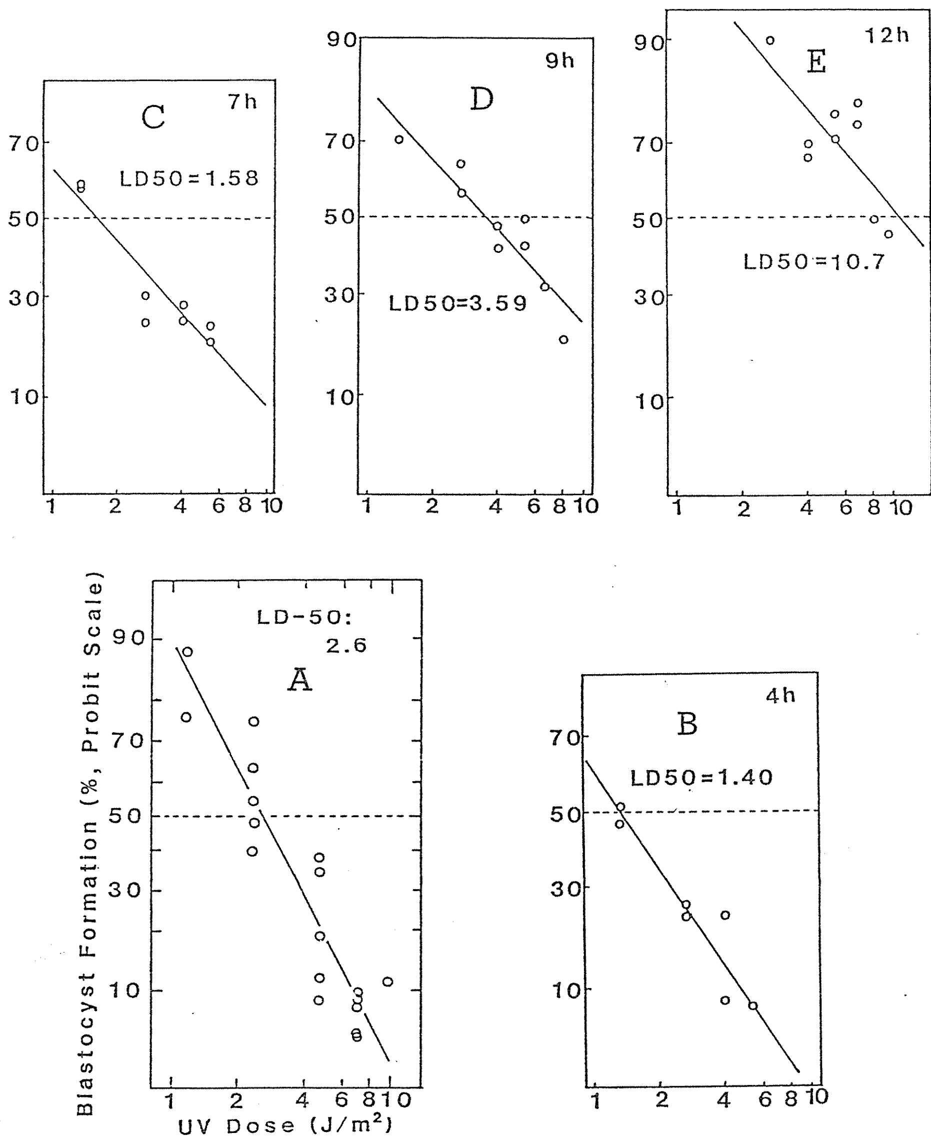


Fig. 1. Determination of half lethal UV fluence (LD-50).

A: Sperm was irradiated with various UV doses indicated on abscissa and then fertilized with the normal eggs. Blastocyst formation of the eggs was examined after 3 days' culture.

B to E: The zygotes were irradiated with UV light at the time after insemination indicated at upper-right portion of the figures. The regression lines were drawn by a computer-programmed calculation.

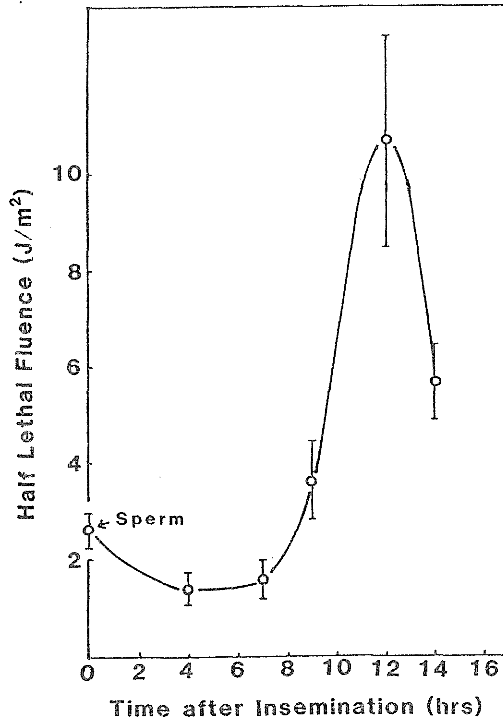


Fig. 2. UV sensitivity variation of the zygotes fertilized in vitro during the pronuclear stage. The zygotes were irradiated with UV light at the times indicated on abcissa and then allowed to develop in vitro to blastocyst stage. The half lethal fluence (LD-50) values were determined as shown in Figs. 1A to 1E. (Data for 14 hr zygotes were not shown in Fig. 1). Bars represent 95 % confidence limits. The first chromosome formation and cleavage division begin at about 15 and 17 hr respectively after insemination.

Early developmental arrest of the UV-irradiated zygotes

Fig. 3A shows the normal expanded blastocyst developed from the in vitro fertilized zygotes. When the zygotes were exposed to UV light, their immediate response was a delay in cleavage, which was severe in the embryos exposed at the early pronuclear stage. Then severely damaged embryos became arrested at the stage earlier than blastocyst stage, degenerated and exhibited abnormal features shown in Fig. 3B. As can be seen in the figures, however, there is a striking difference in the arrested pictures between UV-irradiated (Fig. 3B) and HTO β irradiated (Fig. 3C)

embryos. When the zygotes were exposed to HTO β particles or X-rays, a few embryos died during the cleavage period, but the majority usually continued to develop for several divisions before becoming arrested at the morula stage (1) (Fig. 3C). In the case of UV-irradiated embryos, however, considerable number of the damaged embryos were arrested at earlier cleavage stage such as 2-cell (indicated by arrow b in Fig. 3B) or 1-cell (arrow c) stage.

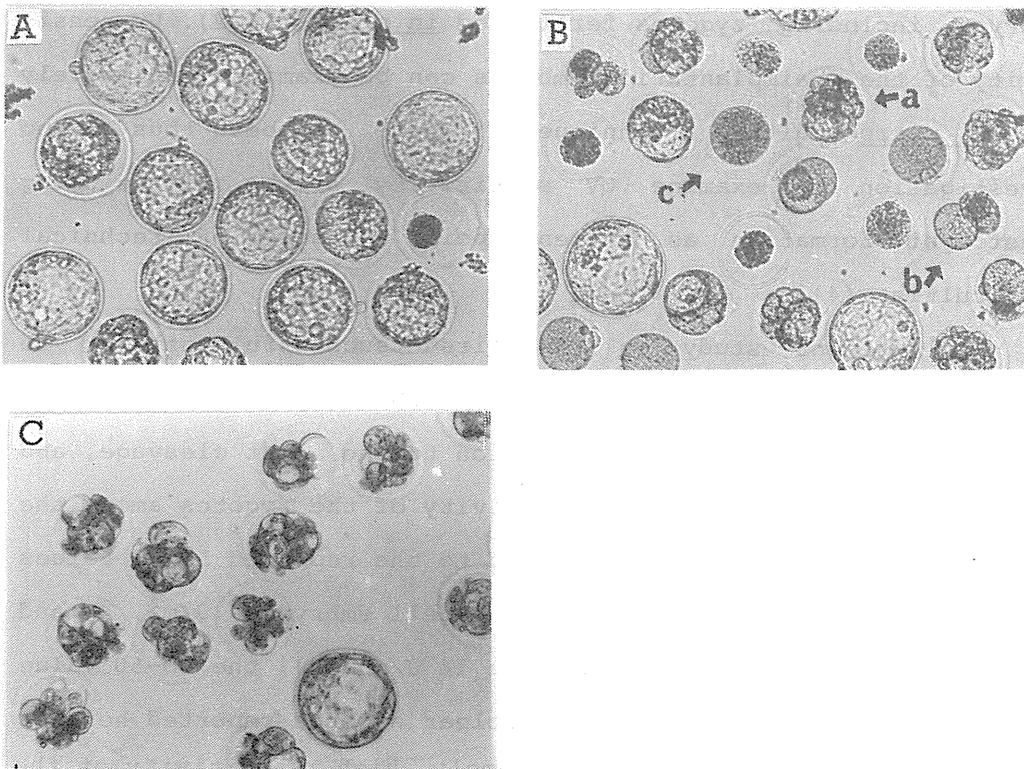


Fig. 3. Normal expanded blastocysts (A) developed from the zygotes fertilized in vitro; abnormal development of the embryos irradiated with UV light at zygote stage (B); abnormal embryos treated with HTO from pronuclear stage (C). Almost all abnormal embryos were arrested at the morula stage when the embryos were treated with HTO from the zygote stage. In contrast to Fig. 3C, different abnormal pictures of the embryos can be noticed in Fig. 3B, such as the embryos indicated by arrows b and c near one of the embryos arrested at the morula stage (arrow a).

DISCUSSION

The effect of X irradiation on preimplantation mammalian embryos depends both on the dose of X irradiation and on the developmental stage of the embryos. The radiosensitivity was determined to be the highest at the zygote stage. With continued development radiosensitivity decreased. An identical sensitivity pattern was obtained after X irradiation in vitro and subsequent in vitro culture of various stages of preimplantation mouse embryos including zygotes fertilized in vitro (1, 2). UV sensitivity of the preimplantation embryos can be examined exclusively by in vitro culture techniques. There has been thus far no investigation to examine UV sensitivity of the zygotes using blastocyst formation as an end point because of technical difficulties (4).

The present study is the first successful attempt to determine the half lethal UV fluence (LD-50) for the zygotes at the various phases from insemination to the first cleavage, and confirmed the highest UV sensitivity of the zygotes among the preimplantation embryos. As compared to the reported LD-50 values for the cultured mouse embryos; 2-cell embryos 9 J/m^2, 4- and 8-cell embryos 10 J/m^2, and morulae 14 J/m^2 (5), the LD-50 value of 1.4 J/m^2 for the early pronuclear zygotes reported here is extremely low, indicating the extremely high sensitivity of the pronculear zygotes.

The present study reveals furthermore that UV sensitivity of zygotes varies markedly depending on the time of irradiation, and pattern of the sensitivity change is exactly similar to the effect of X-rays. The result is in contrast to the different responses of somatic cells to UV-light and X-rays (3). Somatic cells are resistant to UV light in G_1, sensitive through S and

most sensitive in the middle of DNA synthesis; sensitivity then decreases in G_2 (3). Pronuclear DNA synthesis begins about 5 hr after pronuclear formation, which is approximately 7 to 9 hr following sperm penetration, and the S period is about 4 hr (5). If the zygotes used in the present study have the similar cell cycle pattern, Fig. 2 suggests that the zygotes are most sensitive in late G_1, and most resistant in late S to UV light. Investigation of cell cycle progression of the zygotes under the conditions used is needed to discuss further the sensitivity change in connection with the cell cycle.

Another interesting finding of the present study is the differential picture of the dead embryos between UV and ionizing radiations (Figs. 3B and 3C). The embryos which fail to survive after UV irradiation tended to stop proliferating during earlier cleavage stages such as the 2- or 1-cell stage as shown in Fig. 3B, whereas the X- or HTO β-irradiated zygotes can divide more than two times and so embryos which fail to survive stop proliferating mostly at the morula stage (Fig. 3C). After UV irradiation of the 2-cell mouse embryos, there was no indication that a majority of embryos which fail to survive stop proliferating during any particular early stage (7). Further quantitative analysis of cell division number is required to confirm the differential mode of death between UV irradiated and X-irradiated zygotes.

ACKNOWLEDGMENTS

We wish to thank Dr. H. Matsudaira for his encouragement throughout the work and Prof. J. D. Regan for reading and correcting the manuscript.

REFERENCES

1. T. Yamada, O. Yukawa, K. Asami and T. Nakazawa, Effect of chronic HTO or ^{60}Co radiation on preimplantation mouse development in vitro. Radiat. Res. 92, 359-369 (1982).
2. T. Yamada, O. Yukawa, Y. Matsuda and A. Ohkawa, Changes in radiosensitivity of the in vitro fertilized mouse ova during zygotic stage from fertilization to first cleavage. J. Radiat. Res. 23, 450-456 (1982).
3. A. Eddington, Implications of cell division cycle studies. In The Cell Division Cycle Temporal Organization and Control of Cellular Growth and Reproduction (D. Lloyd, R. K. Poole and S. W. Edwards, Eds.), pp. 419-442. Academic Press, New York, 1982.
4. R. A. Pedersen and L. S. Goldstein, Detecting mutations expressed during early development of cultured mammalian embryos. Genetics, 92, s143-s151 (1979).
5. H.-G. Eibs and H. Spielmann, Differential sensitivity of preimplantation mouse embryos to UV irradiation in vitro and evidence for postreplication repair. Radiat. Res. 71, 367-376 (1977).
6. F. W. Luthardt and R. P. Donahue, Pronuclear DNA synthesis in mouse eggs. An autoradiographic study. Exp. Cell Res. 82, 143-151 (1973).
7. E. Endoh and M. Domon, The action of ultra-violet light on the cell proliferation of two-cell mouse embryos. Photochem. Photobiol. 38, 545-550 (1983)

NIRS-M-52, pp 193 - 206, 1985

Chromosomal Effects of Tritium in Mouse Zygotes Fertilized *In Vitro*

Y.Matsuda, T.Yamada[1] and I.Tobari

Divisions of Genetics and Biology[1]
National Institute of Radiological Sciences
9-1, Anaga 4-chome, Chiba-shi 260, Japan

ABSTRACT

The induction of chromosome aberrations in mouse eggs at the pronuclear stage with exposure to HTO β- and ^{60}Co γ-rays was examined by using an *in vitro* fertilization technique. The eggs at pronuclear stage were exposed to β-rays in chemically defined medium containing HTO for 2 hr at 3 to 5 hr after insemination. The eggs at the same stage were exposed to chronic γ-rays from ^{60}Co during the same period. The frequencies of chromosome aberrations increased linearly with dose and the dose-response relationships for frequencies of chromosome aberrations per egg fitted well to a linear equation in both cases. The chromosome aberrations were mainly chromosome-type, and the majority of aberrations were fragments. RBE values of HTO β-rays relative to ^{60}Co γ-rays and X-rays were 1.8 and 1.5, respectively.

INTRODUCTION

Ever since the early work of Russell and Montgomery (1) on the differences in radiation sensitivity during early zygote stages of the mouse, a number of studies have shown that the radiosensitivity varies depending on the pre-implantation stages of the mouse embryo (2,3,4,5). In these studies, the embryos collected from pregnant females were irradiated and cultured _in vitro_ to the blastocyst stage. Such sensitivity changes in the zygote stage at the time of exposure to X-rays was not determined precisely. Yamada _et al_. (6,7) established an efficient culture system to allow eggs to be fertilized _in vitro_ and grown to the expanded blastocyst stage. With this system, we recently examined the radiosensitivity of mouse germ cells just before fertilization, that is, sperm and ova, and zygotes during the period just after fertilization _in vitro_ by observing the retardation of blastocyst formation and chromosome aberration induction at first cleavage metaphases. We found that zygotes at the early pronuclear stage (4 - 6 hr after insemination) had the highest radiosensitivity while mature sperm was relatively radioresistant (7,8,9,10). However, unlike such studies on sensitivity to acute irradiation, there have been few investigations on the response of cultured mouse embryos to chronic radiation, such as tritium β-particles.

The cytological analysis of first cleavage metaphase of fertilized eggs permits direct analysis of transmittable genetic damage which occurrs in germ cells or DNA lesions induced in early stage after fertilization. In the present study, we investigated the sensitivity of fertilized eggs at the pronuclear stage, which is the most sensitive stage to X-rays, to HTO β-particles as measured by cytological analysis of first cleavage

metaphase. Parallel experiments were done with ^{60}Co γ-rays to estimate RBE of HTO β-particles relative to ^{60}Co γ-rays.

MATERIALS AND METHODS

Animals

$BC3F_1$ [$(C57BL/6J \times C3H/He)F_1$] female and RFM male mice were used throughout the experiments. All animals were maintained on a light schedule of 12:00 to 24:00 hr, and were given food and water *ad lib*.

In vitro Fertilization and Embryo Culture

Fertilization of mouse eggs *in vitro* and subsequent cultures of the zygotes were carried out as described in detail previously (6). Two-to four-month-old females were superovulated by intraperitoneal injection of 7.5 IU pregnant mares' serum gonadotropin (PMSG) at 23:00 followed by 7.5 IU human chorionic gonadtropin (hCG) 48 hr later. Fifteen hours after hCG administration mice were sacrificed and tubal eggs were obtained. Sperm had been obtained from the cauda epididymis of three-to five-month-old RFM male mice and capacitated by incubation with fertilization medium at 37°C for 1.5 hr. After capacitation, the sperm suspension was added to the medium containing eggs to give a final sperm concentration of 200 sperms $/mm^3$. At 5 hr after insemination, the fertilized eggs, which extruded the 2nd polar bodies, were washed twice and transferred to the culture medium.

HTO β-Particle Exposure

This was accomplished by the addition of the HTO-containing medium to the culture microdrops at 3 to 5 hr after insemination

to give a final concentration of 500, 1000, 1500 and 2000 μCi/ml. The level of HTO in the culture medium was determined with a liquid scintillation counter. The doses in rad corresponding to given tritium levels were computed on the assumption that the mouse embryo contains about 70% water. From tritium in their medium at 1 μCi/ml, the embryos received a dose of $0.7 \times 0.291 = 0.204$ rad/day (11). Hence the dose rate corresponding to the nominal tritium levels used in this study (500 to 2000 μCi/ml) were 4.25 to 17 rad/hr.

^{60}Co γ-Ray Exposure

Chronic γ-ray exposure at 3 to 5 hr after insemination was carried out as follows. The incubator in which the embryos were being cultured was placed in the field of a ^{60}Co source (approximately 50 Ci) at a distance giving embryo doses in rad corresponding to the tritium levels given above. Dose rates at the microdroplet for the cultures were determined by LiF thermoluminescence dosimeters(TLD) which were calibrated at this institute, and placed in the microdroplet. The incubator remained in the γ-ray beam throughout the incubation period.

Chromosome Preparation

After treatment with colcemid (0.1 μg/ml) at 14 - 18 hr after insemination, the eggs were pooled in a hollow slide containing 1 ml of cold MEM medium supplemented with fetal calf serum and transferred to another hollow slide containing 40% FCS as hypotonic solution. After 25 - 30 min of hypotonic treatment at room temperature, the eggs were transferred to the bottom of hollow slide with freshly prepared fixative (3 : 1 : 4 ethyl alcohol : glacial acetic acid : distilled water) chilled with ice. After 4 min for fixation, a few eggs were

transferred to another fresh fixative for 10 - 20 sec, then set on a clear glass slide and put on a hot plate at 42°C until drying was complete. The slide was immersed in absolute ethyl alcohol for 30 min and stained with 2% Giemsa solution in 0.07M phosphate buffer (ph 6.8) for 5 min.

RESULTS

The results of cytological analysis of first cleavage metaphase of the eggs with exposure to HTO β-rays and ^{60}Co γ-rays at pronuclear stage are summarized in Tables 1 and 2. The structural aberrations observed are shown in Fig. 1. In the controls, some structural aberrations were seen but no numerical aberrations were found, indicating that the hormonal treatment

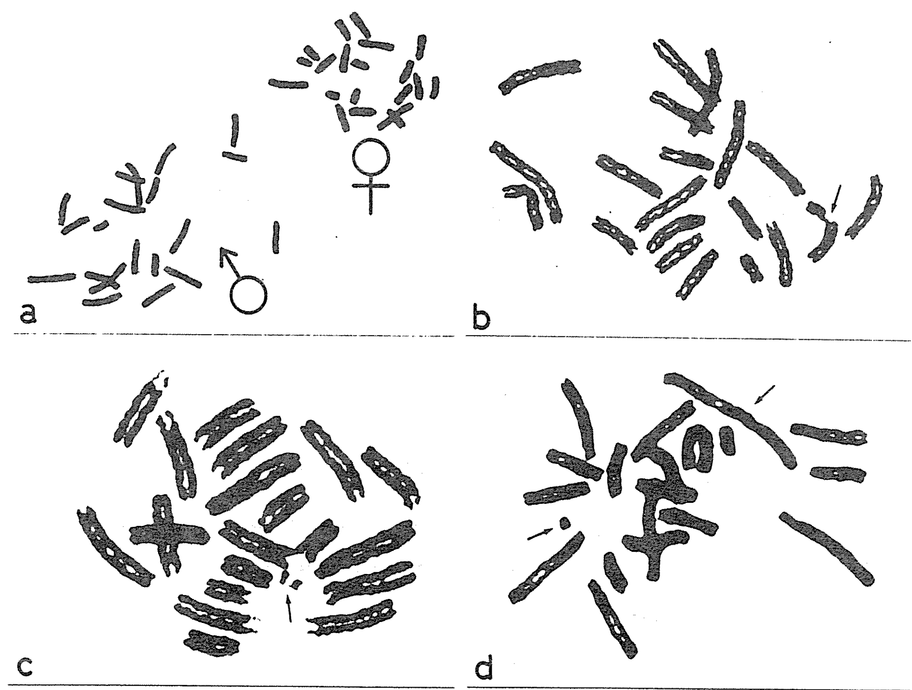


Figure 1. Normal first cleavage metaphase (a) and radiation-induced chromosome aberrations (b-d): (b) chromatid break, (c) chromatid exchange, and (d) chromosome exchange (long chromosome and derived short chromosome)

TABLE I

Frequencies of chromosome aberrations in first cleavage metaphase of zygotes after exposure to HTO β-rays

Exposure (μCi/ml)	No. of metaphase observed	No. of metaphases with chromosome aberrations (%)	No. of chromosome aberrations (per egg)	No. of chromosome aberrations: Chromatid type: Gap and break	Minute	Exchange	Chromosome type: Gap and break	Fragment	Exchange	No. of polyspermic eggs 3n (%)
0	223	2 (0.9)	2(0.009)	2	0	0	0	0	0	17 (7.6)
1000	288	32 (11.1)	34(0.118)	4	0	0	8	22	0	17 (5.9)
2000	292	56 (19.2)	61(0.209)	1	1	0	11	47	1	19 (6.5)
3000	247	81 (32.8)	92(0.372)	4	0	3	10	72	3	5 (2.0)
4000	261	113 (43.3)	125(0.479)	2	2	6	9	105	1	5 (1.9)

TABLE II

Frequencies of chromosome aberrations in first cleavage metaphase of zygotes after exposure to ^{60}Co γ-rays

Exposure (rad)	No. of metaphase observed	No. of metaphases with chromosome aberrations (%)	No. of chromosome aberrations (per egg)	No. of chromosome aberrations: Chromatid type: Gap and break	Minute	Exchange	Chromosome type: Gap and break	Fragment	Exchange	No. of polyspermic eggs 3n (%)
0	223	2 (0.9)	2(0.009)	2	0	0	0	0	0	17 (7.6)
5.2	301	22 (7.3)	23(0.076)	2	0	2	7	11	1	9 (3.0)
19.5	276	36 (13.0)	40(0.145)	5	1	0	3	30	1	19 (6.9)
29.5	216	50 (23.1)	57(0.264)	5	0	2	6	44	0	5 (2.3)

to induce superovulation did not cause significant level of non-disjunction.

In both experiments with exposure to HTO β- and ^{60}Co γ-rays, the frequencies of chromosome aberrations per egg increased linearly with dose. Fig. 2 shows the dose-response relationships for chromosome aberrations per egg, which fitted well to the linear equation, $Y = (-0.14 \pm 1.52) \cdot 10^{-2} + (1.40 \pm 0.07) \cdot 10^{-2} D$ in the experiment with exposure to HTO β-rays and $Y = (1.58 \pm 1.92) \cdot 10^{-2} + (0.79 \pm 0.11) \cdot 10^{-2} D$ in exposure to ^{60}Co γ-rays, where Y is the yield of chromosome aberrations per egg, and D the dose in rad. The RBE value of HTO β-rays relative to ^{60}Co γ-rays calculated from equation of the dose-response for chromosome aberrations induced at the pronuclear stage was 1.77 in the dose range used.

The types of chromosome aberrations observed in the eggs after exposure to HTO β-rays were mainly chromosome-types (92.0%),

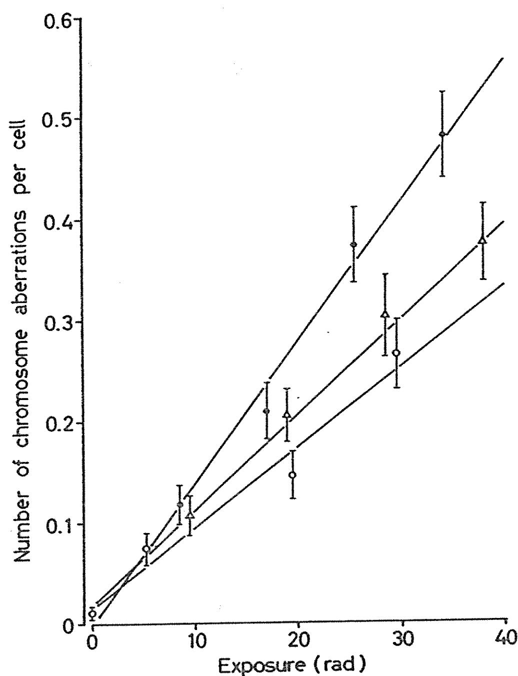


Figure 2. The frequencies of chromosome aberrations after 2 h tritium β-ray (●), 2 h ^{60}Co γ-ray (○) and acute X-ray (△) exposures to eggs at pronuclear stage.

in which the frequency of fragments was especially high (85.1%). The distribution and frequencies of chromosome aberrations observed in eggs after exposure to ^{60}Co γ-rays were similar to the results of experiments with exposure to HTO β-rays (chromosome types: 84.4%, fragments: 82.5%). No rings were observed.

DISCUSSION

This experiment is the first attempt to analyse chromosome aberrations induced by HTO β- or ^{60}Co γ-ray exposure in the early mouse embryo by using the *in vitro* fertilization system. The results of cytological analysis of first cleavage metaphase of eggs with exposure to HTO β- or ^{60}Co γ-rays at pronuclear stage for 2 hr (at 3 to 5 hr after insemination) are summarized

as follows: (1) The eggs at pronuclear stage were very sensitive to both HTO β- and ^{60}Co γ-ray exposures and the frequencies of induced chromosome aberrations increased linearly with dose, the dose-response relationships fitted well to linear equation (Fig. 2). (2) The types of chromosome aberrations observed were mainly chromosome-types, in which the frequency of fragments was the highest (Tables 1 and 2). (3) RBE for HTO β-particles relative to ^{60}Co γ-rays was 1.77.

Previously we investigated the radiosensitivities of mature sperm, mature oocytes and fertilized eggs at pronuclear stage after X-irradiation by cytological analysis of first cleavage metaphase, and showed that the three stages stand in order of radiosensitivity as follows: sperm ⟶ oocytes ⟶ eggs at pronuclear stage (8,9,10). This pattern of sensitivity variation to induction of chromosome aberrations was consistent with that of retardation of blastocyst formation (7), indicating that there is a close relationship between embryonic death and chromosome aberrations induced by radiations. Furthermore, the cytological results in these studies showed that the various types of aberrations including both chromatid- and chromosome-types were observed, and the frequencies of chromosome aberrations were comparable with the frequencies of embryonic retardation before blastocyst stage. These results indicated that the pre-implantation loss might probably be due to all types of chromosome aberrations observed in the first cleavage metaphase. Furthermore, the radiosensitivity of pronuclear stage is extremely high as compared with cultured somatic cells from mammals and humans. Hence the cytological analysis of first cleavage metaphase of eggs at pronuclear stage is available for risk estimation of radiations.

The types of chromosome aberrations were mainly chromosome-types in the both experiments with exposure to HTO β- and ^{60}Co γ-rays. Especially the frequency of fragments was extremely high. Similar tendency was also observed in the fertilized eggs with acute X-irradiation to mature sperm, mature oocytes and pronuclear stage (9,10). However in these experiments, including the present one, chromosome-type exchanges were distinguished by a longer/shorter marker chromosome, so there is a possibility that the frequency of the exchanges might be underestimated, indicating that there might be a bias in estimating chromosome-type aberrations versus chromatid-type aberrations. We are carrying out an experiment in which the frequency of chromosome-type exchanges is estimated correctly. The types of chromosome aberrations induced by HTO were the same as those induced by external low LET radiations including ^{60}Co γ-rays. This result strongly suggests a similarity between DNA damage induced by HTO and external radiations. Therefore, it seems safe to assume that the major effects of tritium on the induction of chromosome aberrations at pronuclear stage are primarily due to the β-rays emitted from tritium oxide.

Bocian _et al_. (12) and Hori and Nakai (13) examined dose-response relationships for chromosome aberrations induced in human lymphocytes by HTO. According to Bocian _et al_. (12), the yields of chromosome-type aberrations fitted into linear-quadratic function, while they followed essentially linear kinetics when the cells were exposed continuously to HTO. On the other hand, Hori and Nakai (13) found in the experiment with continuous exposure for 48 hr that the dose-response curve for the yields of chromatid breaks were biphasic, because very low-dose exposure

induced more aberrations than would be expected from an extrapolation of the linear dose-response seen at higher dose range. Fig. 3 shows the dose-response relationships of chromosome-type deletions induced in the eggs at pronuclear stage with exposure to acute X-rays and chronic HTO β- and ^{60}Co γ-rays (present data, 10). In the experiment of 2 hr exposure to HTO at the G_0 stage of lymphocytes by Bocian _et al_. (12), the yields of deletions, as well as those of dicentrics and rings, gave the best fit to the linear-quadratic functions. On the contrary, in the present experiment of 2 hr exposure to HTO at pronuclear stage of fertilized eggs, the yields best fitted to linear equation, $Y = (-1.78 \pm 1.53) \cdot 10^{-2} + (1.20 \pm 0.07) \cdot 10^{-2} D$ in HTO β-ray exposure, and $Y = (-0.21 \pm 1.17) \cdot 10^{-2} + (0.66 \pm 0.07) \cdot 10^{-2} D$ in ^{60}Co γ-ray exposure. Furthermore, the frequency of chromosome aberrations induced by HTO in the eggs at pronuclear stage is significantly higher than those in lymphocytes. These results

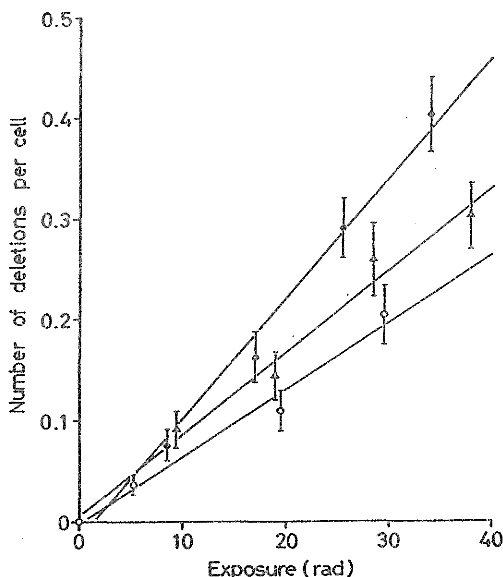


Figure 3. The frequencies of chromosome-type deletions after 2 h tritium β-ray (•), 2 h ^{60}Co γ-ray (o) and acute X-ray (△) exposures to eggs at pronuclear stage.

indicated that the mechanism of chromosome aberration induction by exposure to HTO at pronuclear stage might be different from that in somatic cells. The mechanism of high radiosensitivity in eggs at pronuclear stage remains to be resolved.

The mean RBE values of tritium β-rays as compared with acute 250 kV X-rays (60 rad/min) and chronic ^{60} γ-rays were 1.47 and 1.77 from dose-response curves for total chromosome aberrations, and 1.48 and 1.82, from chromosome-type deletions, respectively (present data, 10). The RBE values were higher than 1.2 and 1.17 reported in Chinese hamster cells (14) and human lymphocytes (12), respectively, and rather close to those in germ cells reported for killing of primary spermatocytes (2.4 and 1.6), killing of primary oocytes (1.6 and 2.0), testis mass loss (1.4 and 2.1) or specific locus mutations (2.2) (11,15, 16,17). The noticeable finding was that the present RBE values of tritium β-rays relative to ^{60} γ-rays on chromosome aberrations were clearly higher than the value (1.09) obtained in the previous study on retardation of blastocyst formation (6). The latter value (1.09) was calculated on the basis of the results of the experiment of 4 days' "continuous" exposure from the pronuclear stage through the blastocyst stage, so it was essentially different from the value (1.77) in the former experiment of 2 hr exposure to HTO. In the experiment of Yamada _et al_. (6) there are various complicated factors such as a number of DNA replications and cell divisions until blastocyst stage, whereas in the present experiment, the eggs were exposed to HTO at presynthetic phase corresponding to G_1 stage only for a short term. Tritium RBE based on chromosome aberrations induced in eggs at pronuclear stage was comparable rather to those on other endpoints reported in germ cells

than those in somatic cells.

ACKNOWLEDGEMENTS

We thank Miss T. Utsugi and Miss A. Ohkawa for their helpful assistance in this experiment. We also wish to acknowledge Mr. F. Nagasawa for his kind supply of experimental animals, and Dr. T. Maruyama and Miss K. Nishizawa for dosimetry of ^{60}Co γ-rays.

REFFERENCES

1. L.B. Russell and C.S. Montgomery, Radiation-sensitivity differences within cell-division cycles during mouse cleavage. Int. J. Radiat. Biol. 10, 151-164 (1965).
2. L.S. Goldstein, A.K. Spindle, and R.A. Pedersen, X-Ray sensitivity of the preimplantation mouse embryo *in vitro*. Radiat. Res. 62, 276-287 (1975).
3. R.H. DuFrain and A.P. Casarett, Response of the pronuclear mouse embryo to X-irradiation *in vitro*. Radiat. Res. 63, 494-500 (1975).
4. K.Y. Ku and P. Voyted, The effects of UV-light, ionizing radiation and the carcinogen N-ácetoxy-2-fluorenylacetamide on the development *in vitro* of one- and two-cell mouse embryos. Int. J. Radiat. Biol. 30, 401-408 (1976).
5. M. Domon, Radiosensitivity variation during the cell cycle in pronuclear mouse embryos *in vitro*. Cell Tissue Kinet. 15, 89-98 (1982).
6. T. Yamada, O. Yukawa, K. Asami, and T. Nakazawa, Effect of chronic HTO or ^{60}Co γ radiation on preimplantation mouse development *in vitro*. Radiat. Res. 92, 359-369 (1982).
7. T. Yamada, O. Yukawa, Y. Matsuda, and A. Ohkawa, Changes in

radiosensitivity of the *in vitro* fertilized mouse ova during zygotic stage from fertilization to first cleavage. *J. Radiat. Res.* 23, 450-456. (1982).

8. Y. Matsuda, T. Yamada, and I. Tobari, Preliminary study on chromosomal aberrations in eggs of mice fertilized *in vitro* after X-irradiation. *Mutation Res.* 121, 125-130 (1983).
9. Y. Matsuda, T. Yamada, and I. Tobari, Studies on chromosome aberrations in the eggs of mice fertilized *in vitro* after irradiation. I. Chromosome aberrations induced in sperm after X-irradiation. *Mutation Res.* in press.
10. Y. Matsuda, I. Tobari, and T. Yamada, Studies on chromosome aberrations in the eggs of mice fertilized *in vitro* after irradiation. II. Chromosome aberrations induced in mature oocytes and fertilized eggs at pronuclear stage after X-irradiation. in preparation.
11. R.L. Dobson and T.C. Kwan, The RBE of tritium radiation measured in mouse oocytes: Increase at low exposure levels. *Radiat. Res.* 66, 615- 625 (1976).
12. E. Bocian, B. Ziemba-Zak, O. Rosiek, and J. Sablinski, Chromosome aberrations in human lymphocytes exposed to tritiated water *in vitro*. *Curr. Top. Radiat. Res.* 12, 168-181 (1977).
13. T. Hori and S. Nakai, Unusual dose-response of chromosome aberrations induced in human lymphocytes by very low dose exposure to tritium. *Mutation Res.* 50, 101-110 (1978).
14. W.C. Dewey, R.M. Humphrey, and B.A. Jones, Comparisons of tritiated thymidine, tritiated water and cobalt-60 gamma-rays in inducing chromosome aberrations. *Radiat. Res.* 24, 214-238 (1965).

15. B.E. Lambert, Cytological damage produced in the mouse testes by tritiated thymidine, tritiated water and X-rays. Health Phys. 17, 547-577 (1969).

16. T.E.F. Carr and J. Nolan, Testis mass loss in the mouse induced by tritiated thymidine, tritiated water, and ^{60}Co gamma irradiations. Health Phys. 36, 135-145 (1979).

17. W.L. Russell, R.B. Curmming, E.M. Kelly and E.L. Phipps, IAEA-SM-232/85. International Symposium on the Behavior of Tritium in the Environment. San Francisco, California. (1978).

NIRS-M-52, pp 207 - 220, 1985

Tritium Effects on the Gonads of the Aquarium Fish, *Oryzias latipes*. 1. Fecundity and Fertility

Y.Hyodo-Taguchi and H.Etoh

Division of Biology
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

To determine effects of early radiation-induced germ cell loss on life-time reproduction, the fecundity and fertility of the fish given chronic irradiation in their embryonic stages were investigated. Embryos of medaka, *Oryzias latipes*, were exposed to tritiated water(0.05-2.0 mCi/ml) and ^{137}Cs γ-rays (6.1-254 rad/day) continuously from the morula stage until hatching(10 days at 26°C). Newly hatched fry were removed from the radioactive environment. At 4-8 months after hatching pairs consisting of irradiated males and nonirradiated females or the reverse were mated. On the basis of the daily yield of eggs per pair, fecundity and fertility of the irradiated fish were estimated. When irradiated females were mated with unirradiated males, both the total number of oviposition and the number of eggs per fish decreased with increasing exposure-rate.

However, most eggs laid were fertilized and hatched normally in all groups. Tritium β-rays were more effective than ^{137}Cs γ-rays in reducing the fecundity of irradiated females. In the irradiated male pair groups, the total number of oviposition hardly decreased, but the number of fertilized eggs per fish decreased depending on the dose-rate . There was little difference of effectiveness of β-rays and γ-rays in decreasing the fertility of the irradiated males.

INTRODUCTION

In a series of experiments designed to study tritiated water β-ray effects on fish primordial germ cells, the present authours have reported that the dose response of the germ cells showed an exponential relationship when embryos of medaka were treated with tritiated water at a concentration of 0.2 mCi/ml or lower. In fry treated at a concentration of 0.4 mCi/ml or more, on the other hand, the number of germ cells remained almost constant at approximately 30(1). In order to determine effects of early radiation-induced germ cell loss on life-time reproduction, the fecundity, or the number of offspring produced per female, and fertility of male fish given chronic irradiation with β-rays from tritiated water and γ-rays from ^{137}Cs in their embryonic stages were investigated.

MATERIALS AND METHODS

Experiments were carried out at 26°C with fish of the r/R strain of medaka, Oryzias latipes, which were the offspring from the closed colony given by Dr. H. Tomita, Nagoya University. The fertilized eggs were kept in tritiated water at concentrations of 0.05 to 2 mCi/ml(Table I) within 2 hr after fertilization.

To prevent cross-contamination and to trap the tritiated water vapor, a closed cabinet system was employed as described previously (1). To avoid accidental contamination, renewal of the culture media was done inside a fume hood. The radioactivity in culture media was measured by a liquid scintillation counter(Beckmann 7500) after dilution to appropriate concentrations. Dose rate of β-rays from tritium incorporated into the egg was calculated with formula $D=A \cdot E \cdot K/100\ W$, where D is dose rate in rad per minute, A, disintegrations per minute, E, average β-ray energy in keV(5.69), K, 1.602×10^{-9}(erg/keV), W, weight of an egg(g) and 100, factor to convert erg/g to rad. In this calculation, the radioactivity(A) in egg was estimated according to Ueno by using a concentration factor of 0.60(3).

The fertilized eggs were also irradiated continuously with ^{137}Cs γ-rays at dose-rates of 6.1 to 254 rad/day until hatching at different distances(47 to 290 cm) from a source of 10 Ci(Table I). Dose absorbed by the eggs was determined by an IONEX-250 dosimeter with a conversion factor of 0.95. Irradiation of 22 hr a day was achieved by an automatic controller. Total dose for 22 hrs was represented as daily dose(rad/day).

In all experiments culture media for the eggs were renewed each day and dead eggs were removed. The fry hatched in 9 to 11 days. The newly hatched fry were removed from the radioactive environment and kept in plastic vessels containing 3 liters of water. Fry were given a powdered fish food(Tetramin, Tetra Werke Co., Melle, Fed. Rep. Germany) and *Daphnia*. Under these conditions, fry developed to sexual maturity within 2-3 months. At 4 to 8 months after hatching, fish were tested for their fecundity and fertility. Matured fish laid eggs almost every morning. A lot of pairs consisting of irradiated males and nonirradiated

females or the reverse were mated from each irradiated group (Table I). Eighty-four pairs of unirradiated males and females served as control. Each pair was kept in a separate vessel and clusters of eggs laid were collected every morning. Within several hours after fertilization, eggs were examined under the dissection microscope and separated into the normal(fertilized) which developed to the morula stage and the abnormal which consisted of unfertilized eggs and a small number of dead one. Observations were performed during the successive 14 days with each pair.

RESULTS

Table I shows the number of fish examined for fecundity and fertility together with the data on the accumulated dose during the period of 10 days in embryonic development. Eighty-four pairs consisting of unirradiated females and males served as controls. Three hundred thirty seven irradiated females and 365 irradiated males in total were examined daily for yield of eggs. Figure 1 shows the typical daily yield of eggs per pair during a 14-day period. In each dose group, the daily yiely of eggs in 1st to 5th individuals selected at random was drawn on the graph. All pairs in controls laid eggs almost every day, 13 of a 14-day period. A cluster of eggs consisting 10-30 were laid. When females treated with tritiated water were mated with unirradiated males, the number of females laying eggs decreased with increasing dose. None of the 9 females in 2 mCi/ml laid any eggs for the entire testing period. In the 1 mCi/ml group, one of the 21 females laid eggs for 13 consecutive of a 14-day period. Ten of the 21, 18 of the 31, 7 of the 13, 19 of the 31 and 13 of the 17 females laid eggs almost every day in 0.5, 0.2, 0.15, 0.1 and 0.05 mCi/ml group,

respectively. On the basis of daily yield of eggs per pair changes in the fecundity and fertility of the fish following exposure to chronic β- and γ-rays at their embryonic stages were shown in Figs. 2 and 3.

A dose-dependent reduction in the fecundity of the irradiated females expressed in the number of oviposition was observed when they were mated with unirradiated males, but most eggs in each cluster were found to be fertilized. Consequently, little difference was noted between total oviposition frequency and

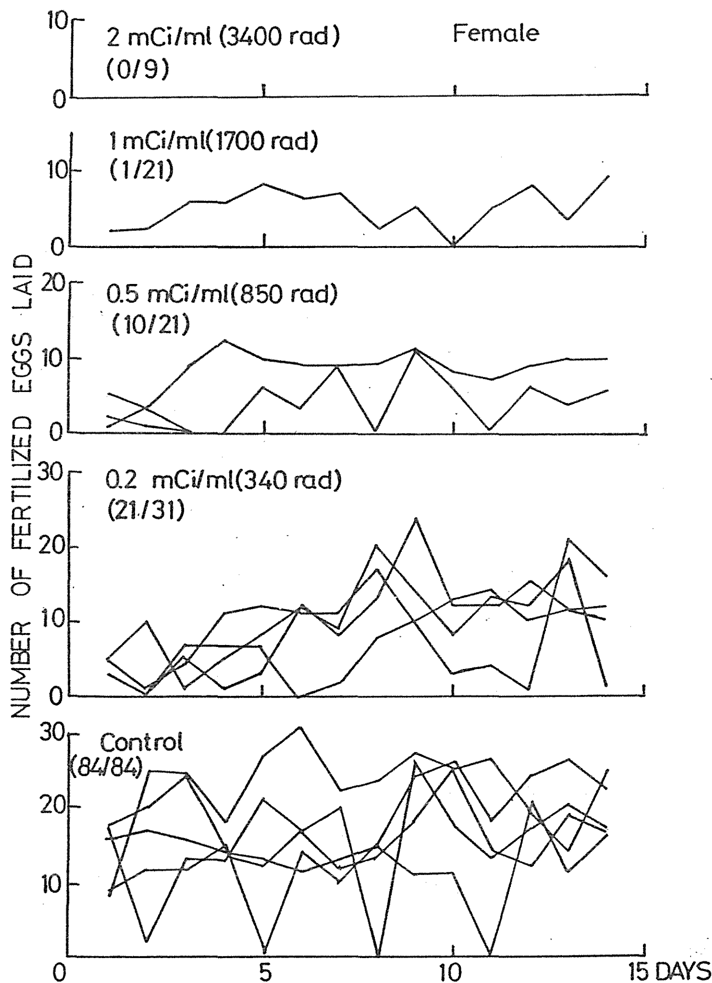


Fig. 1 Effects of tritiated water on daily yield of eggs per pair during a 14-day period. Females were treated at their embryonic stages.

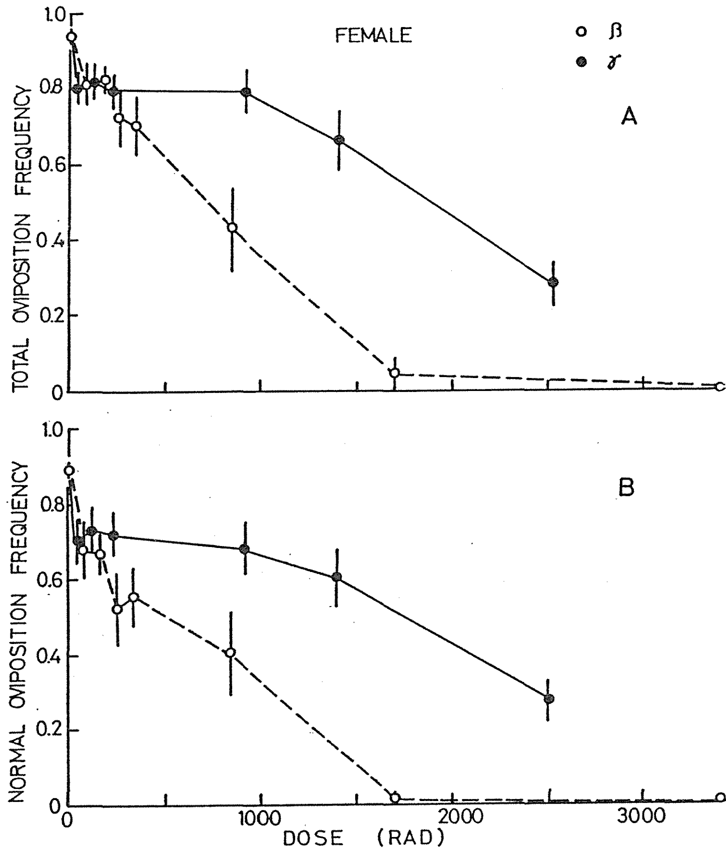
TABLE I

Scheme of Treatment for Embryos and Number of Fish Examined for Fertility and Fecundity

	Concentration or distance	Accumulated dose for 10 days(rad)	Number of fish examined Female	Male
Control	0	0	84	84
HTO	0.05 mCi/ml	85	17	22
β-rays	0.1	170	30	15
	0.15	255	13	12
	0.2	340	31	31
	0.5	850	21	19
	1.0	1700	21	31
	2.0	3400	9	9
^{137}Cs	290 cm	61	20	21
γ-rays	220	105	21	16
	153	240	42	58
	72	910	31	25
	55	1400	27	40
	34	2540	54	66

normal oviposition frequency(Fig. 2). The total oviposition frequency and normal oviposition frequency are the ratios of the number of ovipositions and the number of ovipositions with fertilized eggs to the total number of ovipositions examined. Tritium β-rays were more effective than ^{137}Cs γ-rays in reducing the oviposition frequencies in irradiated females.

When irradiated males were mated with unirradiated females to examine fertility, the total oviposition frequency hardly decreased(Fig. 3A). However, the normal oviposition frequency markedly decreased depending on the dose(Fig. 3B). No clear differences were found in the effectiveness of β-rays and γ-rays in decreasing the oviposition frequency, contrary to irradiated females. This finding indicates that the mating behavior of irradiated males is not affected by irradiation in spite of germ cell loss. The number of completly infertile fish, the pair which did not lay any fertilized eggs during the experimental period, increased depending on the dose.

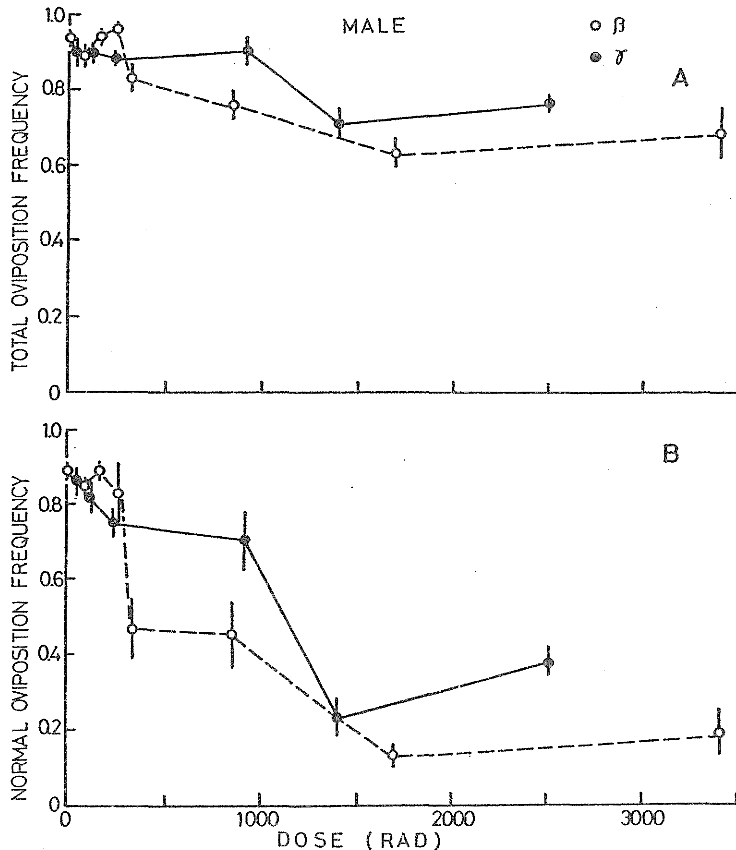


$$\text{TOTAL OVIPOSITION FREQUENCY} = \frac{\text{TOTAL NUMBER OF OVIPOSITION}}{\text{NUMBER OF OVIPOSITION TESTED}}$$

$$\text{NORMAL OVIPOSITION FREQUENCY} = \frac{\text{NUMBER OF OVIPOSITION WITH FERTILIZED EGGS}}{\text{NUMBER OF OVIPOSITION TESTED}}$$

Fig. 2 Fecundity of female medaka exposed to β- and γ-rays at their embryonic stage. A; Total oviposition frequency, B; Normal oviposition frequency.

Figure 4 shows the changes in the number of eggs per oviposition in the irradiated fish. Both in the irradiated females and males, a clear dose dependent reduction was found in the number of fertilized eggs, althought the total number of eggs laid from the pair of irradiated males and unirradiated females decreased little. In the low dose range of 0 to 400 rad, distinct reduction in the number of fertilized eggs was observed in both sexes but no differences were found between β- and γ-rays. At doses of 500 rad or more, on the other hand,



$$\text{TOTAL OVIPOSITION FREQUENCY} = \frac{\text{TOTAL NUMBER OF OVIPOSITION}}{\text{NUMBER OF OVIPOSITION TESTED}}$$

$$\text{NORMAL OVIPOSITION FREQUENCY} = \frac{\text{NUMBER OF OVIPOSITION WITH FERTILIZED EGGS}}{\text{NUMBER OF OVIPOSITION TESTED}}$$

Fig. 3 Fertility of male medaka exposed to β- and γ-rays at their embryonic stages. A; Total oviposition frequency, B; Normal oviposition frequency.

the number of fertilized eggs laid was reduced less abruptly in γ-irradiated than β-irradiated fish.

To compare the effects on reproductive capacity of tritium β-rays with those of ^{137}Cs γ-rays, the ratios of the mean number of eggs laid per oviposition in irradiated fish to that in controls were plotted against dose on a semi-logarithmic scale (Fig. 5). A nonlinear relationship was obtained and there was no evidence of a threshold in diminishing the reproductive capacity of each sex for both types of radiations. No differences

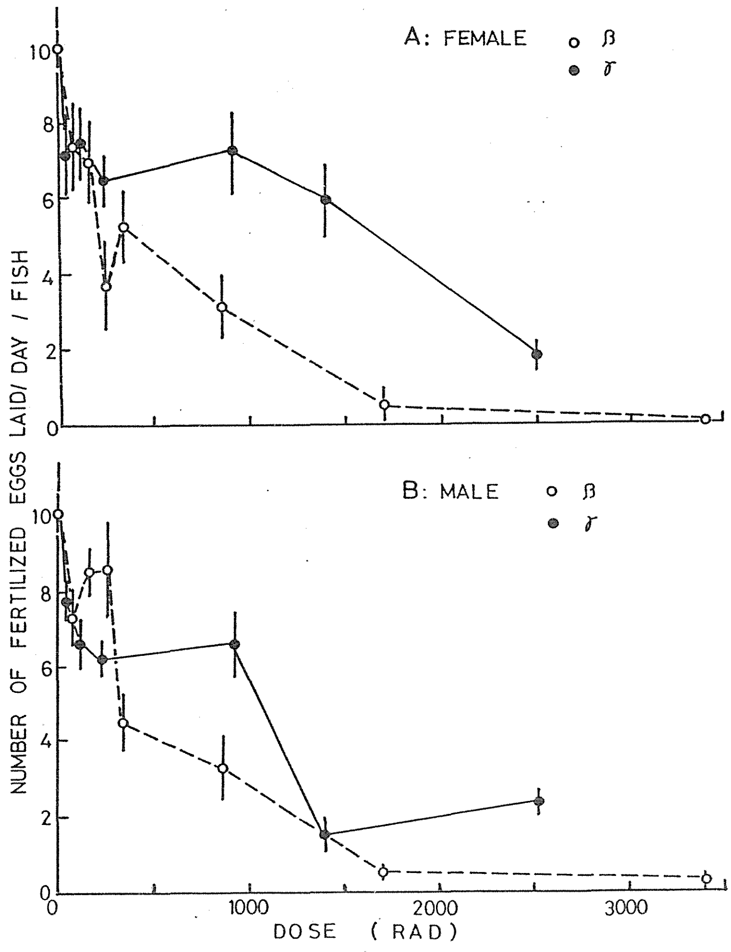


Fig. 4 Number of fertilized eggs per oviposition from medaka exposed to β- and γ-rays at their embryonic stages. A; Female irradiated, B; Male irradiated.

due to radiation quality were obtained up to 250 and 500 rad in females and males, respectively. Above 500 rad, however, the curves started to separate and at higher dose range β-irradiation became more effective than γ-irradiation. Especially, in females, the γ-ray curve inflected suddenly at about 250 rad and its slope became less thereafter.

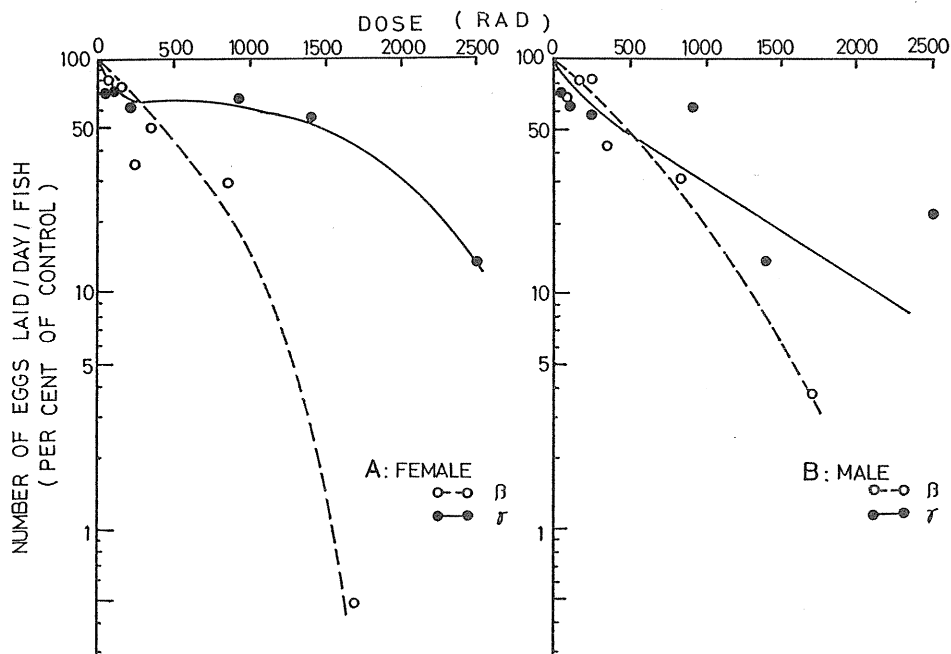


Fig. 5 Dose-response relationship of reproductive capacity in medaka exposed to β- and γ-rays at their embryonic stages. A; Female irradiated, B; Male irradiated.

DISCUSSION

The loss of the primordial germ cells in medaka embryos has been reported by several investigators after exposures to γ-rays from ^{137}Cs or β-rays from ^{3}H during embryogenesis(1, 3-6). The irradiated embryos differentiated into either males or females and they showed clearly the secondary sexual characters. In the present experiments, almost proper differentiation of irradiated embryos to females or males were observed. Furthermore, no effects on the mating behavior of males were observed after embryonic exposure to chronic irradiation. The total oviposition frequency remained undiminished as in the previous paper(7).

It is possible to test the ability of the primordial germ cells to differentiate into functional gametes, ova or sperms, by changes in the fecundity or fertility of the fish. A dose-depend-

ent diminution of reproductive capacity were found in this experiment by testing fecundity and fertility of the chronically irradiated fish. There was no evidence of a threshold in the reduction of reproductive capacity of each sex for both types of radiations. In males, the loss of reproductive capacity seems to reflect the exponential diminution of primordial germ cells in chronically irradiated fry at low doses. The 50 % survival doses for male germ cells at hatching were 390 rad for β-rays and 520 rad for γ-rays(8). In the present experiments, the doses 50 % loss of male reproductive capacity were around 500 rad both for β- and γ-rays. In female, however, chronic irradiation effects were less on reproductive capacity than on the loss of germ cells at hatching. The 50 % survival doses for female germ cells at hatching were 140 rad for β-rays and 305 rad for γ-rays(1). The accumulated dose for 50 % loss of female reproductive capacity is 400 rad for tritium β-rays and at higher doses, no tailing is observed in dose response curve for β-rays. In γ-irradiated female, moreover, an inflection was observed in the dose-response curve at about 250 rad, the dose of about 40 % loss, followed by a plateau and markedly high dose(1500 rad) was necessary for 50 % loss of the reproductive capacity.

It is very difficult to explain these peculiar results. They may be due to the differences of proliferation kinetics in female and male germ cells just after hatching. Around hatching time, successive mitoses take place in the female germ cells and just after hatching some of them begin to enter into the meiotic prophase. During this period, however, the male germ cells are mitotically dormant and about 15 days after hatching they begin to divide. The first spermatocytes and spermatids can be observed

60 days after hatching. If we assume a high repair ability for female germ cells and low or no ability for male germ cells just after hatching, a small number of female germ cells probably survive after γ-irradiation and regenerate rapidly, but after tritiated water treatment, a low but significant residual radioactivity in important component of the cells may preclude the regeneration of the germ cells survived.

In mammals, several studies of the effects of continuous low-level HTO exposure on developing female germ cells and on succeeding generation(F_1 litter size, weight loss of various organs of offsprings, and dominant lethal mutations) have been reported(9-14). Irreparable oocyte loss is induced by low dose of tritiated water in mice(9, 10). The effective dose causing 50 % reduction in the oocyte number is approximately 2 μCi/ml of HTO in body water, corresponding to 6 rad(10). In this workshop, effects of early radiation-induced oocyte loss on life-time reproduction have been reported by Dr. Dobson: Reproduction was less affected than germ-cell loss and there was a threshold dose observed in the reduction of reproductive capacity because of large pool of the ovarian germ cells. It has been shown from involving experiments intrauterine exposure to tritiated water that a dose of 3 rad per day, associated with body-water tritium level of 10 μCi/ml, appears to be a critical radiation dose for reproductive function in the rat(11-14).

The present results suggest no evidence of a threshold in the reduction of reproductive capacity of female and male fish following chronic exposure to tritiated water within the dose range examined although fish germ cells are less sensitive to tritium than mammalian germ cells. To examine the genetic effects following chronic exposures to tritiated water, experiments

have now been started; the results will be reported elsewhere.

ACKNOWLEDGMENTS

This work is supported by a grant for project research on Biological Effects of Tritium from the Science and Technology Agency.

REFERENCES

1. H. ETOH and Y. HYODO-TAGUCHI, Effects of tritiated water on germ cells in medaka embryos. Radiat. Res.93, 332-339(1983).
2. A. H. UENO, Incorporation of tritium from tritiated water into nucleic acids of *Oryzias latipes* eggs. Radiat. Res. 59, 629-637(1974).
3. S. HAMAGUCHI, Differential radiosensitivity of germ cells according to their developmental stages in the teleost, *Oryzias latipes*. In Radiation Effects on Aquatic Organisms (N. Egami, Ed), pp.119-128. Japan Sci. Soc. Press, Tokyo/ Univ. Park Press, Baltimore(1980).
4. N. EGAMI and A. HAMA-FURUKAWA, Late effects of continuous γ-irradiation of the developmental stage of the gonads in *Oryzias latipes*. ibid. pp.105-117(1980).
5. N. EGAMI and A. HAMA-FURUKAWA, Response to continuous γ-irradiation of germ cells in embryos and fry of the fish, *Oryzias latipes*. Int. J. Radiat. Biol. 40, 563-568(1981).
6. K. IJIRI and N. EGAMI, Effects of γ-ray irradiation on primordial germ cells in embryos of *Oryzias latipes*. Radiat. Res. 72, 164-173(1977).
7. Y. HYODO-TAGUCHI and H. ETOH, The fecundity and fertility of medaka exposed to chronic γ-radiation in their embryonic stages. J. Radiat. Res. 24, 270-277(1983).

8. H. ETOH and Y. HYODO-TAGUCHI, Effects of β-rays from tritiated water on germ cells in medaka embryos and fry. In Proceedings of the Seventh International Congress of Radiation Research(J. J. Broerse, G. W. Barendsen, H. B. Kal and A. J. van der Kogel, Eds), Martinus Nijhoff Publishers, Hague, C3-07(1983).

9. R. L. DOBSON and M. R. COOPER, Tritium toxicity: Effect of low-level ^{3}HOH exposure on developing female germ cells in the mouse. Radiat. Res. 58, 91-100(1974).

10. R. L. DOBSON and T. C. KWAN, The RBE of tritium radiation measured in mouse oocytes: Increase at low exposure levels. Radiat. Res. 66, 615-625(1976).

11. D. F. CAHILL and C. L. YUILE, Tritium: Some effects of continuous exposure in utero on mammalian development. Radiat. Res. 44, 727-737(1970).

12. J. W. LASKEY, J. L. PARRISH, and D. F. CAHILL, Some effects of lifetime parental exposure to low levels of tritium on the F_2 generation. Radiat. Res. 56, 171-179(1973).

13. R. J. HAAS, W. SCHREML, T. M. FLIEDNER and W.CALVO, The effects of tritiated water on the development of the rat oocyte after maternal infusion during pregnancy. Int. J. Radiat. Biol. 23, 603-609(1973).

14. A. L. CARSTEN and S. L. COMMERFORD, Dominant lethal mutations in mice resulting from chronic tritiated water(HTO) ingestion. Radiat. Res. 66, 609-614(1976).

NIRS-M-52, pp 221 - 230, 1985

Tritium Effects on the Gonads of the Aquarium Fish, *Oryzias latipes*. 2. Histological Changes

H.Etoh and Y.Hyodo-Taguchi

Division of Biology
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

Histological changes of the gonads of adult medaka irradiated with ^{3}H β-rays or ^{137}Cs γ-rays during their embryogenesis were studied. After measurement of the weight of fish body and their gonads, the gonads were observed microscopically on histological sections. Weights of irradiated gonads were decreased with increasing doses of β-rays. After γ-irradiation, marked decrease in weight of gonads was found in females irradiated with 2500 rad and in males with 1400 and 2500 rad. Embryos irradiated within 250 rad developed into fertile fish. Rate of fertile fish was decreased to less than approximaltely 10% after β-irradiation of 1700 rad or more, whereas 30% or more of the fish were still fertile after γ-irradiation of 2500 rad.

From the present and previous results, it seems that the recovery by germ cell repopulation is dominant in females, although the primordial germ cells at embryonic stages are more sensitive to radiation in female than in male, and that the decrease in gonad weight is not observable within a certain dose range which induce, however, reductions in the number of offsprings.

INTRODUCTION

The effects of tritiated water on germ cells in medaka embryos have been studied (1, 2). From the comparison of changes of the number of germ cells between medaka irradiated with ^{3}H β-rays and ^{137}Cs γ-rays during their embryogenesis, the β-rays were found more effective to kill the female germ cells than γ-rays. The male germ cells, however, were affected in similar manner by both types of radiations. The data also showed that the female germ cells were more sensitive than male germ cells to two radiations.

The changes in the fecundity and fertility of the medaka given chronic irradiation of ^{137}Cs γ-rays in their embryonic stages and the effects of ^{3}H β-rays relative to ^{137}Cs γ-rays on these end points have also been reported (3, 4).

In the present experiments, gonad-somatic ratios and histological changes of the gonads in the same adult medaka that were used for the tests of fecundity and fertility were studied.

MATERIALS AND METHODS

Materials used in the experiments were the same fish that were used for the tests of fecundity and fertility (4). Procedures of irradiation with β-rays and γ-rays of the embryos of medaka (Oryzias latipes) and calculation for doses were presented in previous papers (1, 4). Briefly, the eggs were kept in tritiated water at concentrations of 0.05 to 2 mCi/ml or irradiated with ^{137}Cs γ-rays at dose rates of 6.4 to 254 rad/day from morula stage to hatching. Newly hatched fry were removed from the radiation field and kept at 26°C until sexual maturaion. Matured fish were tested for the fecundity and fertility. The medaka were fixed with Bouin's solution at the end of those tests. Weights of fish body and their gonads were measured following the change of the fixative to 70% alcohol. Rate of maturation of fish was described as a change in gonad-somatic ratio (GSR) which was the ratio of weights of the gonad and the body for each fish. After the measurement, those gonads were cut at 6 μm and stained with Mayer's hemalum and eosin for histological observation.

RESULTS

Changes in weights of gonads

Results obtained are shown in Table 1. Weights of the irradiated ovaries and testes decreased generally with increasing doses of β-rays. The GSR of fish irradiated with 1700 rad of β-rays was decreased to approximately 10% of that of non-irradiated fish. Little difference was found between

the GSRs of the control and the fish irradiated with 900 rad or lower of γ-rays, whereas GSRs were decreased when doses of 1400 rad or more for males and 2500 rad for females were given at their embryonic stages. The gonads which weighed less than 0.1 mg were defined as undeveloped gonads in this study.

Table 1. Gonad-somatic ratios (GSRs) of medaka irradiated during their embryonic stages.

		Concentration or distance	Accumulated dose for 10 days (rad)	Male			Female		
				Number of fish tested	GSR ± S.E.	(%)	Number of fish tested	GSR ± S.E.	(%)
Exp. 1	Control		0	17	0.32 ± 0.06	100.0	12	7.72 ± 0.65	100.0
	HTO	0.05 mCi/ml	85	22	0.31 ± 0.05	96.9	16	7.74 ± 0.53	96.4
		0.1	170	15	0.33 ± 0.06	103.1	30	7.12 ± 0.47	92.2
	^{137}Cs	290 cm	61	12	0.42 ± 0.05	131.2	16	6.15 ± 0.64	79.7
		220	105	16	0.25 ± 0.05	78.1	13	6.44 ± 0.79	83.4
Exp. 2	Control		0	45	0.46 ± 0.04	100.0	38	9.17 ± 0.49	100.0
	HTO	0.15 mCi/ml	225	11	0.31 ± 0.06	67.4	11	7.58 ± 1.04	82.7
		0.2	340	31	0.20 ± 0.04	43.5	31	4.77 ± 0.66	52.0
		0.5	850	18	0.36 ± 0.09	78.3	21	4.60 ± 1.11	50.2
		1.0	1700	30	0.07 ± 0.02	15.2	21	0.35 ± 0.33	3.9
		2.0	3400	10	0.03 ± 0.04	6.5	8	0.03 ± 0.003	0.3
	^{137}Cs	153 cm	238	36	0.47 ± 0.05	102.2	32	8.77 ± 0.76	95.6
		72	908	15	0.47 ± 0.08	102.2	15	7.76 ± 0.88	84.6
		55	1400	26	0.17 ± 0.04	37.0	12	7.65 ± 1.07	83.4
		34	2544	61	0.21 ± 0.03	45.7	48	2.99 ± 0.67	32.6

The number of fish having undeveloped gonads is shown in Table 2. Most embryos irradiated with 250 rad or lower developed into fertile fish. When embryos were irradiated with 250 rad or more of both types of radiations, the number of fish having undeveloped gonads was gradually increased in both sexes. Rate of fertile fish was decreased to less than approximately 10% after β-irradiation of 1700 rad or more, whereas 30% or more of the fish were still fertile after γ-irradiation of 2500 rad.

Table 2. Appearance of the fish having undeveloped gonads.

	Accumulated dose for 10 days (rad)	Number of fish tested	Number of fish having undeveloped testes	Per cent of fertile fish	Number of fish tested	Number of fish having undeveloped ovaries	Per cent of fertile fish
Control	0	62	0	100	50	0	100
	85	22	0	100	16	0	100
	170	15	0	100	30	0	100
HTO	255	11	2	82	11	0	100
(β-rays)	340	31	12	61	31	11	65
	850	18	8	56	21	11	48
	1700	30	26	13	21	20	5
	3400	10	10	0	8	8	0
	61	12	0	100	16	0	100
	105	16	0	100	13	0	100
^{137}Cs	238	36	2	94	32	2	94
(γ-rays)	908	15	2	87	15	1	93
	1400	26	17	35	12	2	83
	2544	61	29	53	48	30	38

*Undeveloped gonads were less than 0.1mg of weight.

Histological changes

In non-irradiated fish, the ovary contained a number of oocytes in various developmental stages (Fig. 1A), and the testis contained all spermatogenic cells (Fig. 2A). The gonads of the fish irradiated with various doses of β- and γ-rays are shown in Figs. 1 and 2. Undeveloped gonads were observed in the groups irradiated with 250 rad or more. These gonads were difficult to find with the naked eyes, and therefore, removal of them for the observations was done under a dissecting microscope. A very few primordial germ cell-like cells were seen in the undeveloped gonads (Figs. 1D, 2D). Irradiated testes were small in size and number of spermatogenic cells. Sometimes, male fish with large testes were found regardless of size of radiation dose. The large testes, so called ovo-testes, consisted of male germ cells with various size and a number of oocytes-like cells (Figs.

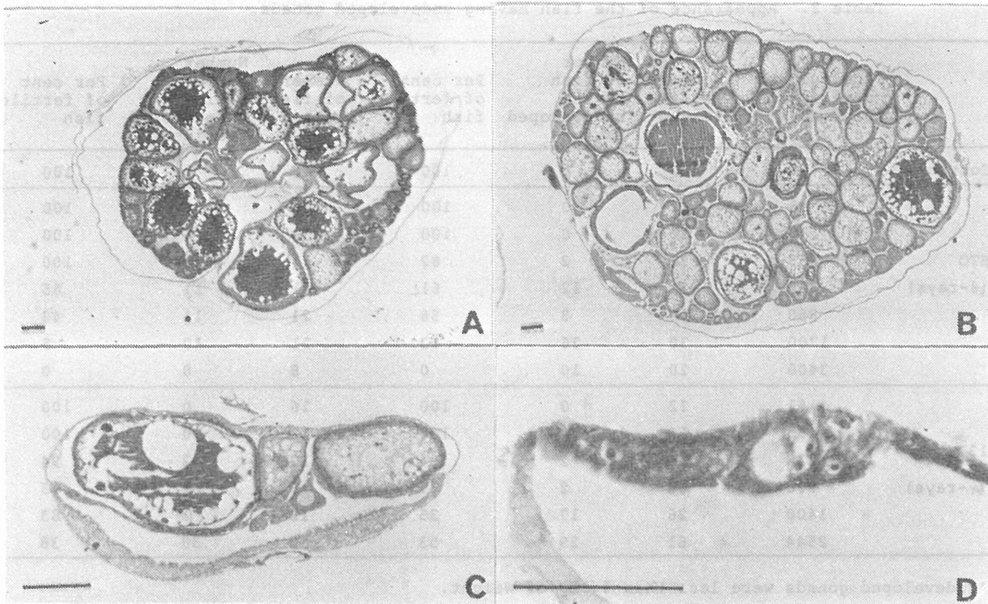


Fig. 1. Cross sections of ovaries of the fish that were irradiated with various doses during their embryogenesis. Bars on the left bottom in each photo show 200μm except 50μm in D. A: A normal ovary. Many oocytes in various developmental stages are seen. This fish laid a total number of 114 eggs for 14 days and 90% of them were normal. B: An ovary of the fish irradiated with 238 radof γ-rays. This fish laid a total of 134 eggs for 14 days and 91% of them were normal. C: An ovary of the fish kept at 0.5 mCi/ml of HTO. A few well developed oocytes were seen. However, the fish laid no eggs at all except 3 abnormal eggs for 14 days. D: An undeveloped ovary of the fish kept at 2 mCi/ml of HTO. The fish laid no eggs for 14 days.

2B, 2C). Figure 2C shows an extreme case of ovo-testis in which germ cells differentiated abnormally into large oocyte-like cells. These abnormal oocyte-like cells may degenerate at a certain stage and never ovulate normally.

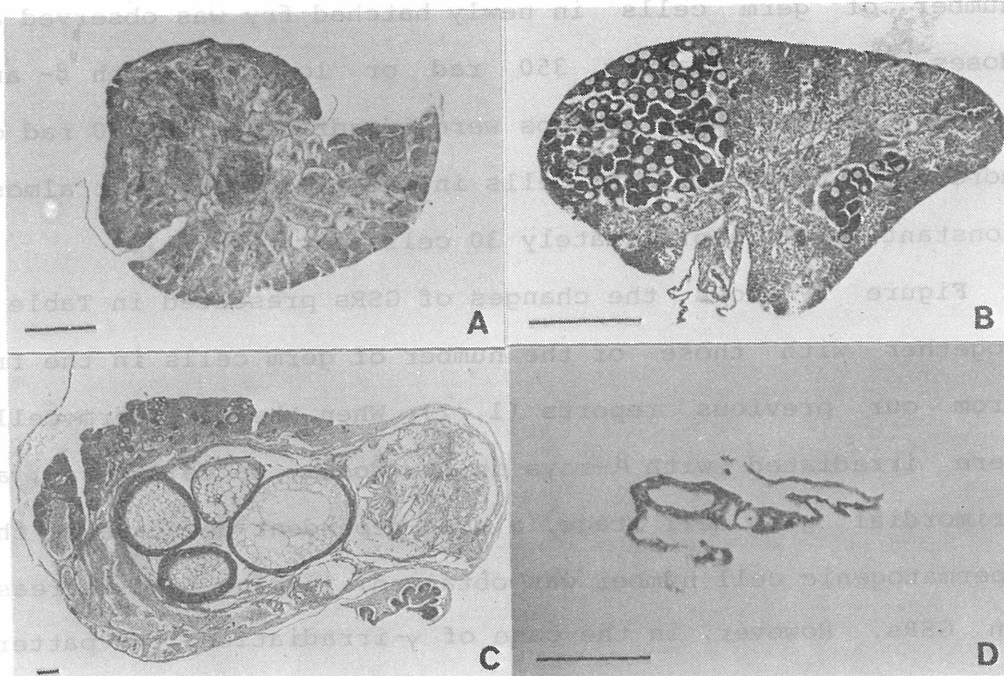


Fig. 2. Cross sections of testes of the fish irradiated with various doses during their embryogenesis. Bars on the left bottom in each photo show 200μm. A: A normal testis. All components of spermatogenetic cells are seen. Mating of this fish produced 209 eggs and 91% of them were normal. B: An ovo-testis of the fish irradiated with 260 rad of γ-rays. A part of the testis was occupied with a large number of oocyte-like cells. This male had only a weak reproductive capacity, about 19% of the eggs being fertilized. C: An ovo-testis in which germ cells are differentiated abnormally into more matured oocyte-like cells and occupied a large part of the testis. The fish was irradiated with 900 rad of γ-rays and produced only 2% fertilized eggs, out of 131 eggs for 14 days. D: An undeveloped testis of the fish that was kept in 2 mCi/ml of HTO. There are a few primordial germ cell-like cells in the testis. This fish produced no fertilized eggs at all.

DISCUSSION

The effects of continuous irradiation of ^{3}H β-rays and ^{137}Cs γ-rays on the developing germ cells have been investigated in medaka (1-4). A dose-dependent decrease in the

number of germ cells in newly hatched fry was observed at doses of approximately 350 rad or lower for both β- and γ-rays. However, when embryos were irradiated with 700 rad or more, the number of germ cells in the fry remained at almost constant level, approximately 30 cells per fish (1).

Figure 3 shows the changes of GSRs presented in Table 1 together with those of the number of germ cells in the fry from our previous reports (1, 2). When the male germ cells were irradiated with β-rays in low doses, up to 350 rad, at primordial germ cell stage, a dose-dependent decrease in the spermatogenic cell number was obtained, judging from decrease in GSRs. However, in the case of γ-irradiation, the pattern of changes of GSRs was different from that of germ cell number in the fry, that is, no clear decrease in GSR was observed up to 900 rad, due possibly in part to the appearance of ovo-testes. In the females, GSRs of fish irradiated with 250 rad were approximately 80% of the control for the two radiations, whereas the germ cell numbers in the fry decreased to 30% after β-irradiation and 50% after γ-irradiation. From the histological observations, it can be considered that ovary weight was recovered by repopulation of the germ cells, although the recovery was weaker in fish irradiated with β-rays than those irradiated with γ-rays. As was shown in Table 2, when the embryos were irradiated with 250 rad or lower of β-rays and with 900 rad or lower of γ-rays, almost of them developed into fertile fish. Thereafter incidence of the undeveloped gonads was increased with increasing doses, that is, the number of fertile fish was

decreased with increasing doses. These findings on the GSR change indicate the presence of a threshold dose beyond which radiation effects on the development of gonads appear.

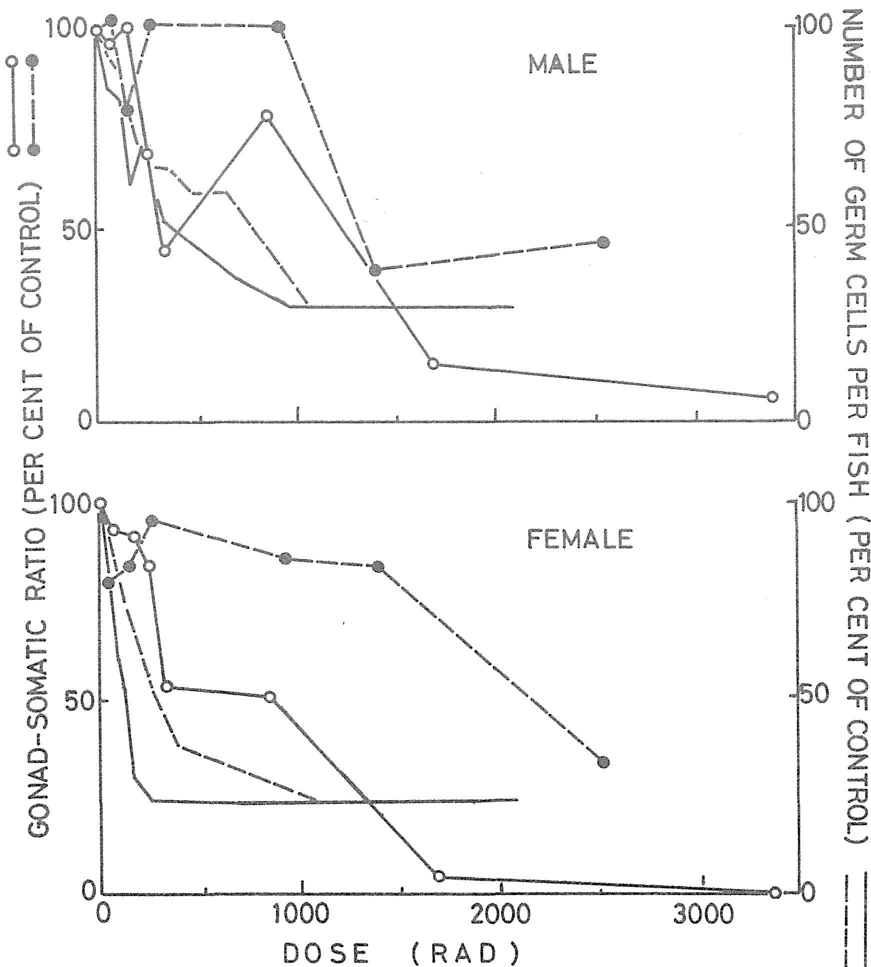


Fig. 3. The changes of gonad-somatic ratios in adult medaka together with the number of germ cells in the fry from our previous papers (1, 2). Solid lines with and without open circles show the GSR and the germ cell number after β-irradiation, respectively. Broken lines with and without closed circles show the GSR and the germ cell number after γ-irradiation, respectively.

From the present and previous results of fecundity and fertility (4), it seems that the recovery by germ cell repopulation is dominant in females, although the primordial

germ cells at embryonic stages are more sensitive to radiation in female than those in male, and that the decreases in gonad weight are not observable within a certain dose range which induce reduction in the number of offsprings.

ACKNOWLEDGMENTS

This work is supported by a grant for profect research on Biological Effects of Tritium from the Science and Technology Agency.

REFERENCES

1. H. ETOH and Y. HYODO-TAGUCHI, Effects of tritiated water on germ cells in medaka embryos. Radiat. Res. 93, 332-339 (1983).
2. H. ETOH and Y. HYODO-TAGUCHI, Effects of β-rays from tritiated water on germ cells in medaka embryos and fry. Abstract on Somatic and Genetic Effects (C3-05), 7th ICRR, Amsterdam, July 3-8 (1983).
3. Y. HYODO-TAGUCHI and H. ETOH, The fecundity and fertility of medaka exposed to chronic γ-irradiation in their embryonic stages. J. Radiat. Res. 24, 270-277 (1983).
4. Y. HYODO-TAGUCHI and H. ETOH, Tritium effects on the gonads of the aquarium fish, *Oryzias latipes*. 1. Fecundity and fertility. Proc. 2nd US-Japan Workshop on Tritium Radiobiology and Health Physics. pp - , (1985).

NIRS-M-52, pp 231 - 232, 1985

Tritium Effects on the Mouse Ovary: Oocyte Killing, Fertility Loss, and Genetic Implications

R.L.Dobson, T.C.Kwan and T.Straume

Biomedical Sciences Division
Lawrence Livermore National Laboratory
University of California, Livermore, CA 94550, U.S.A.

Primordial, "resting" oocytes are the main constituents of the ovarian germ-cell pool and the principal cells at genetic risk in the female. They are formed only before birth, hence lost ones cannot be replaced. In women these cells are not easily killed by radiation; but in mice they are extremely sensitive (LD_{50} = 6 rad). Interestingly, for chronic exposure, beta rays from tritium in body water are more effective than gamma rays in killing mouse oocytes (limiting RBE ~ 3).

Because low ^{3}HOH exposures can cause irreparable oocyte loss in young mice, we examined effects on fertility by measuring lifelong reproductive capacity. Reproduction is less affected than germ-cell number. Even with early oocyte losses of 80-90%, offspring diminish less than 20%. However, initial oocyte deficiencies of only about 50% cause reproduction to cease prematurely. Despite differences between human and mouse oocytes, extrapolation estimates from murine data to man may be attempted with the aid of ^{3}HOH data from monkeys, although uncertainties are large. Premature menopause appears the most likely effect in women - from irradiation before birth - and may possibly follow relatively low exposures.

Of greater significance is the question of sensitivity to mutagenesis. Heretofore, all investigator efforts to find heritable changes induced by radiation in primordial oocytes of mice have failed. This is curious. It has led to the notion (probably erroneous) that these particular cells may somehow be resistant to mutation. However, our

comparisons of ^{3}HOH in body water and ^{3}H-TdR in oocyte DNA (as well as studies with neutrons and gamma rays) show that the super-sensitive lethality target in mouse primordial oocytes is not DNA, but probably the plasma membrane. This offers to explain the negative genetic findings in mice (oocytes that survive receive doses to DNA which are downwardly biased). And it warns of possible under-estimation of genetic risk in the human female. Experiments taking into account the membrane lethality target are now underway (using ^{3}H, ^{125}I, and low-energy neutrons) to detect and quantify mutation induction in these critically important murine cells.

(Work performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-Eng-48.)

NIRS-M-52, pp 233 - 245 , 1985

Tritium - Induced Cell Death in the Intestinal Epithelium of the Mouse

K.Ijiri and T.Shiroya

Zoological Institute
Faculty of Science
University of Tokyo
Hongo, Tokyo 113, Japan

ABSTRACT

Tritiated water (HTO) was injected intraperitoneally into mice of 10 weeks old, the dose per mouse ranging from 0.1 mCi to 100 mCi. Mice were sacrificed 3-24 hours later and the dead cells (apoptosis) in the crypts of small intestine were examined for their yield and positions in the crypt. In the 100 mCi-injected mice the number of dead cells reached a peak value, about 4 cells per crypt section at 6 hours after the administration of HTO. The count decreased slightly at 9 hours and then to the half (2 cells/crypt section) at 24 hours. The groups which received 1.0 and 10 mCi HTO showed a dose-dependent yield at each time of examination. The distributions of dead cells were obtained for HTO-injected mice, with cell death at the same positions in the crypt base as in the mice

irradiated with gamma-rays or given tritiated thymidine injection. This indicated that the toxicity of tritiated water expressed as cell death in the crypts was due to beta-rays from tritium itself. Some characteristics of stem cells in small intestinal crypts and their response to various cytotoxic agents were reviewed.

INTRODUCTION

The epithelium of the small intestine is a rapidly renewing cell population in adult mammals. Cells are continuously produced in the crypts of Lieberkühn. Small-intestinal crypt of mouse ileum consists of about 250 cells, which are arranged as a sheet, one cell thick, moulded into a flask-shaped structure (Fig.1).

The crypt cells in the intestinal epithelium were chosen for the investigation of the effects of tritiated water (HTO) mainly for following reasons. i) Intestinal death is important in the sense that it is one of the three major patterns of death of individual animals, with the other two being hematopoietic and brain death. ii) Recently, it was found that there exist radiosensitive cells in the crypts. This permits a highly sensitive and quantitative assay for the effects at low-dose levels in a very short time after administration of HTO. These sensitive cells exhibit their damage as a rapid cell death, and their number per crypt, D_0 value and their re-establishment kinetics after irradiation have been determined from acute exposure studies (1,2). iii) Such cells hypersensitive to radiation have been regarded as a part of stem cell population in the crypt, and this was also pointed out from the cell position studies of cell death

after various cytotoxic drugs (2,3). HTO gives an essentially uniform beta-ray-irradiation to all crypt cells following its injection into mice. The use of such uniform beta-ray-irradiation enables us to compare the results on cell death with those obtained from external irradiation and localized intracellular beta-rays from ^{3}H-thymidine. This may add informations on stem cell positions and its numbers in the crypts. It also gives the RBE of HTO when the effects of the continuous external irradiation were adequately compared (9).

MATERIALS AND METHODS

Animals

Male C57BL/6 mice aged 10 weeks old (c.a. 25 g) were used. The animals had been kept in an animal room under a 12 h dark (18.00-06.00)-12 h light regimen, and were given food and water *ad libitum*.

Facility for tritium handling

All the experimental procedures from the injection of HTO to mice till the dehydration and embedding of intestine samples, were done in the 'tritium safety clean cabinet', a safety facility designed for the tritium experiments on both cultured cells and animals (Radioisotope Centre, University of Tokyo). Two inner boxes (a kind of glove boxes) with tritium trappers (cooling with dry ice-etahnol) were set up in the cabinet. In one box, most of the procedures were performed such as preparation of the HTO solution for injection, injection to mice and their dissection. The other inner box was used only for the maintenance of HTO-administered mice for various times (3-24 hours). Three mice (one data point) were

placed in a small plastic cage and usually three or four cages were placed in the inner box. The same regimen of light cycle, food and water as in animal room was realized in this box. Flow of air, clean and tritium-free, was sufficiently provided. Details on this facility and its efficiency will be reported elsewhere.

Administration of tritiated water

HTO with a specific activity of 2 Ci/0.4 ml was obtained from Amersham International, U.K.. This was diluted to an appropriate concentration with distilled water and injected intraperitoneally to mice using a microsyringe. Before injection, an aliquot of such HTO solution was further diluted and its radioactivity was determined by a liquid scintillation counter. Each injection volume was kept to about 0.2 ml (ranged between 0.18-0.21 ml). Care was taken that none of the injected solutions escaped after removal of the needles. All injections were performed at 09.00. Control mice received 0.2 ml of distilled water outside the cabinet and were kept in an ordinary animal room.

Sample preparation

At various times (3-24 h) after HTO administration, mice were killed by cervical dislocation. The small intestine was fixed in Carnoy's solution for 30 min prior to storage in 70% of ethanol. During a few weeks, ethanol (70%) was changed several times to reduce the HTO content in the samples as possible. Segments of ileum were embedded in paraffin, sectioned transversely at 5 µm, and stained with hematoxylin and eosin as described previously (3).

Scoring of dead cells (apoptoses) and their distribution in crypts

These techniques have been described previously, together with the histological characteristics of the cell death concerned (1-3). Briefly, the levels of radiation-induced cell death can be assessed by counting the number of cells undergoing nuclear pycnosis or karyorrhexis in histological preparations of crypts. Such dead or dying cells are called as 'apoptosis' (for its detailed definition, see Ref.(4)). Fifty crypt sections were scored for each mouse. The distributions of dead cells (or their fragments, referred as 'apoptotic fragments') in crypt were obtained as follows: Starting at the base of the crypt column the cells were numbered up each side as shown in Fig.1, and the position of cells containing apoptotic fragments was recorded up to the crypt end (the junction with the villus). The results are presented as frequency distributions against cell position. From each distribution, the peak position of cell death (x_{med}) and the two parameters of the spread of the distribution (σ_r

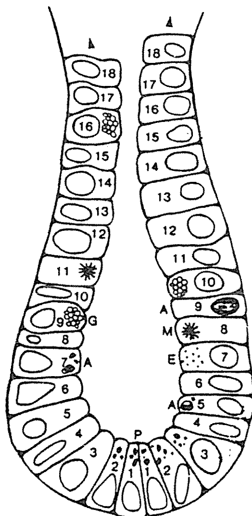


Fig.1. Schematic representation of a small-intestinal crypt showing Paneth cells (P), goblet cells (G), enteroendocrine cells (E), mitoses (M) and apoptosis (cell death, A). Cells are numbered from the base to the top of the crypt. (Ref.(3)).

and σ_1) were calculated as mathematically defined previously (3). Additional comments on the reason for choosing these parameters were referred to elsewhere (see Materials and methods in Ref.(2)).

RESULTS

Number of dead cells

HTO was given (i.p. injection) at a dose per mouse ranging from 0.1 mCi to 100 mCi, and 3,6,9 and 24 hours later the incidence of cell death (apoptotic cells) in the crypts of the small intestine was examined. Figure 2 shows the changes in the number of dead cells per crypt section with time after the injection. When 100 mCi HTO was given, the cell death yield reached a peak value (i.e. about 4 dead cells/crypt section) at 6 h. The yield then decreased slightly at 9 h and then almost to the half (about 2 cells/crypt section) at 24 h. The

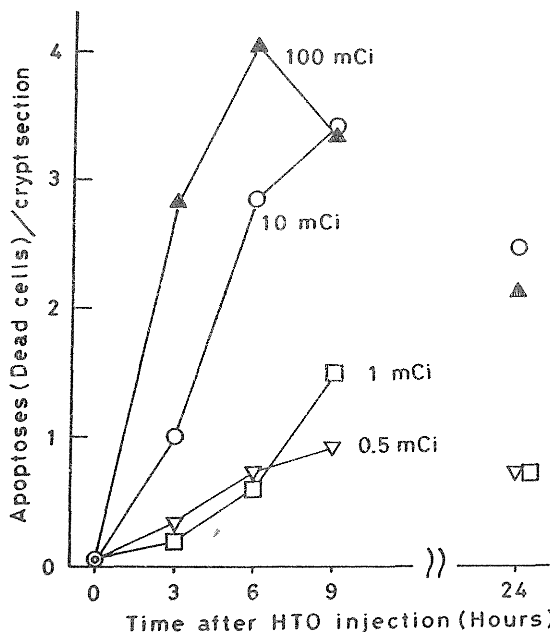


Fig.2. The cell death yields in crypt sections with time after administration of HTO at different doses. Each point represents the mean of determinations on 3 mice except 100 mCi group (2 mice per point).

decrease in count can be attributable to the exhaustion of all the target cells at 6 h, followed only by the digestion of dead cells in the tissue. In all the animals that received 100 mCi, at each time examined, a marked depression in the mitotic activity of crypt cells was observed. At 3 h, mitotic figures decreased to 0.4/crypt section and a frequency of 0.05 or less (/crypt section) was obtained at 9 and 24 h: the value for the untreated control mice was 1.2/crypt section. Shortening of the size of the crypt was also observed in the treated animals.

For those mice given 10 mCi or less, as a general trend, an increased incidence of cell death with increasing time was observed within 3 to 9 h. The data of 0.1 mCi per mouse (the lowest dose given) lay somewhere around the level of the untreated control mice (spontaneous level). More crypts should be examined to determine whether in the group of 0.1 mCi there exists significant level of cell death compared with the control value. The data of 0.5 and 1.0 mCi at 3 and 6 h seemed to show slightly opposite yields, probably within the limit of data fluctuation. However, at 9 h after HTO administration, the incidence was clearly in a range of dose-dependent increase. In general, the cell death caused by HTO is a dose-dependently increasing phenomenon. Experiments are now under way to establish a precise dose-response curve for HTO-induced cell death.

Distributions of dead cells

Distributions of apoptotic fragments (dead cells or their fragments) against each cell position in the crypt are shown in Fig.3, as frequency histograms (normalized to the per-

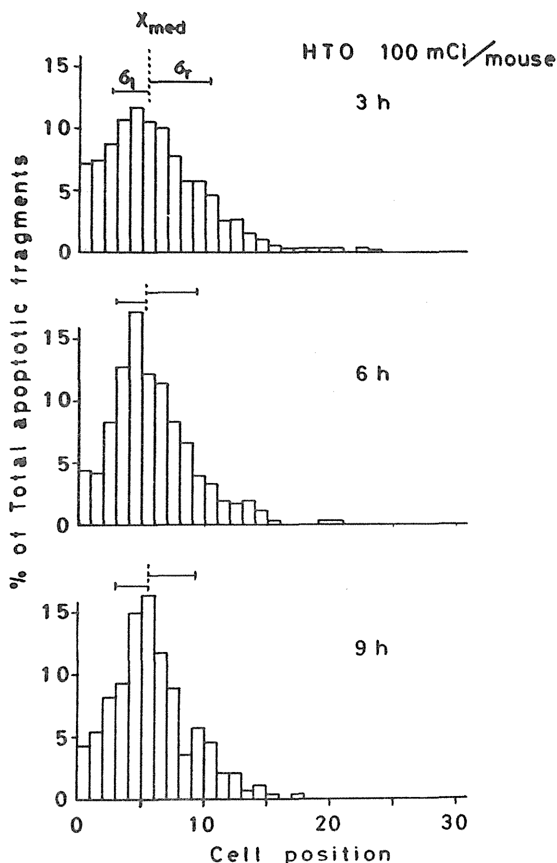


Fig.3. Distribution of cell death at various times after HTO administration. A dose of 100 mCi/mouse was used. Frequency of apoptotic fragments at each cell position is shown as the percentage of the total frequency. On each distribution diagram, three parameters described in the text are shown: The vertical broken line represents the peak position of cell death (x_{med} , the median point), while the horizontal lines show the extent of 6_r and 6_l , which are measures of spread of the right or left halves of the distribution, respectively.

TABLE I

Three Parameters for the Distributions of Cell Death Induced by HTO

Dose per mouse	Hours examined after injection	Peak position (x_{med})	Measure of spread	
			right half (6_r)	left half (6_l)
100 mCi	3 h	5.4	4.9	2.9
	6 h	5.3	4.1	2.3
	9 h	5.5	3.7	2.5
10 mCi	6 h	5.3	4.4	2.9

centage against the total frequency). The distributions for those crypts examined at 3,6,9 h after 100 mCi HTO are presented. Three parameters calculated, i.e. x_{med} (a measure of peak position; the median), σ_r (a measure of the spread of the right half of the distribution; the standard deviation of right half) and σ_l (a similar measure of left half) are indicated on each histogram and summarized also in Table I. The distribution for 10 mCi-injected mice at 6 h was also analyzed. All these four histograms had a peak position (x_{med}) of 5.3-5.5 with a small range of variation. The values for σ_r were 3.7-4.9. Sets of these two values (x_{med} , σ_r) are plotted in Fig.4, together with the summary of published data on cell death induced by various drugs, acute external radiations and tritiated thymidine (^{3}HTdR).

As seen in Fig.4, similar distribution parameters were obtained for the crypts treated with HTO, external radiations and ^{3}HTdR. This indicates that the toxicity of HTO expressed as cell death in the crypts is due to the ionizing radiations (beta-rays) from tritium itself.

DISCUSSION

Hierarchical organization of crypt cells

The cells ultimately responsible for all of the crypt cell population, i.e. 'stem cells' are presumed to lie near the base of the crypt, and all the crypt cells represent an hierarchical cell lineage originating from such stem cells, related with the positions in the tissue architecture of crypt. The real base of the crypt (cell position 1-4) contains several mature and immature Paneth cells. Immediately

above this Paneth zone, i.e. at cell position 5, stem cells are thought to exist, or around this position they are abundant, mostly concentrated at cell position 5. The latter idea may be more likely. These cells cycle at a slower rate (T_c = 24 h) than the majority of proliferating crypt cells situated at cell position 6-20 (T_c = 12 h) (5).

Peak positions of cell death after various cytotoxic agents

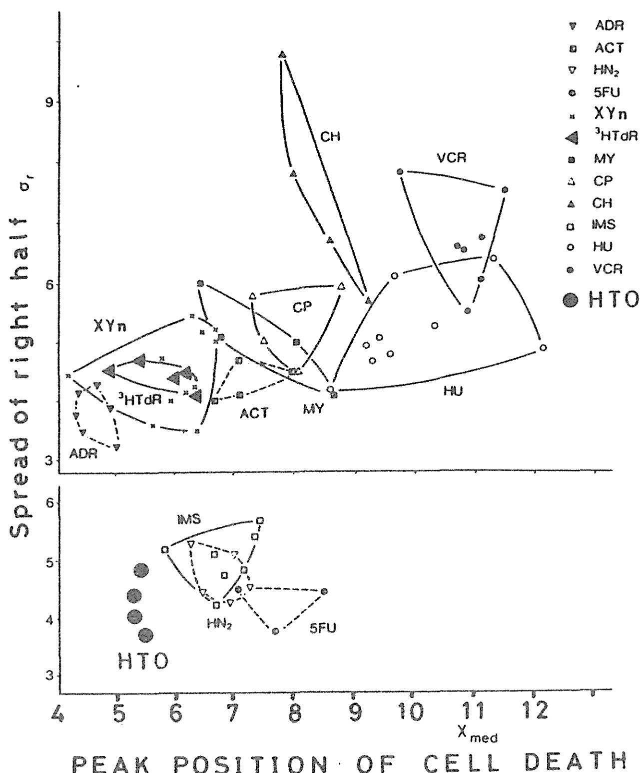


Fig.4. Cytotoxic response of crypt cells at different cell positions to various cytotoxic agents. These data were previously published by Ijiri and Potten (3), to which the data of HTO are added. For each distribution of cell death (apoptotic distribution), the peak position of cell death (x_{med}, the median) and a measure of spread of right half (σ_r) have been calculated and plotted (see text). All the points plotted represent results obtained over the first 12 h after the exposure to various doses of cytotoxic agents. The points for each agent are enclosed by a solid or broken line. The results for 3 agents and that of HTO (the present experiment) are plotted separately on the lower graph for clarity. External radiations (X-rays, gamma-rays, neutrons) are shown as XYn. For the full name of the drugs and more details, see Ref.(3).

Cell death induced by various cytotoxic agents has been recorded for each cell position up the side of the crypts. The results, in the form of distributions of dead cells against each cell position, show that each of the cytotoxic agents tends to act preferentially over a characteristic small range of cell positions. Thus, each cytotoxic agent has some specificity for cells of a particular hierarchical status. A summary of the results on the distributions of cell death caused by many drugs and radiations is shown in Fig.4. All drugs were injected i.p. to mice.

Significance of radiation-induced cell death in the crypt

So far there have been no powerful techniques to determine directly the actual number of stem cells. The number of clonogenic cells, e.g. 80 cells (5) gives us the upper limit of the number of stem cells. From the results of several indirect experiments and insight, 14 or 16 was suggested as the most probable number of stem cells (5,6).

Some cells in the lower regions of the crypt are extremely radiosensitive, being killed by very low doses of acute gamma-ray-irradiation. These sensitive cells are regarded as a part of stem cell population and described as having D_0 on a survival curve as little as 0.1 to 0.2 Gy (1,7). The peak position of the distribution of radiation-sensitive cells, being at cell position 5-6 as seen in Fig.4, corresponds to the position where stem cells are presumably located.

When a low dose (e.g. 0.5 Gy) is given, there is no ablation of whole crypts, but death of sensitive cells can be detected. This induced cell death possibly triggers the recruitment of other stem cells (G_0 or G_1-blocked stem cells)

into rapid cell cycles. Thus, the size of the stem cell population is maintained (8). This local homeostatic regulation within the stem cell compartment itself can be achieved without dramatic changes in the cellularity of the crypt. How rapidly these sensitive cells are re-established after irradiation was also studied (2). The data indicates the presence of other more resistant stem cells (G_0 stem cells) and also implies that radiation-sensitive cells are stem cells which are in cell cycle when irradiated.

ACKNOWLEDGMENTS

This work was supported by the Grant-in-Aid for Fusion Research from the Ministry of Education, Science and Culture. The tritium experiments, though still preliminary, have been carried out as a collaborative study among several members of University of Tokyo, i.e. Drs. N. Morikawa and N. Nogawa, Mrs. M. Ohno and ourselves (K.I. and T.S.). We thank these people for allowing us to present these unpublished data. We are grateful to Miss Takako Hiromasa for technical help.

REFERENCES

1. C.S. Potten, Extreme sensitivity of some intestinal crypt cells to X-and γ-irradiation. Nature 269, 518-521 (1977).
2. K. Ijiri and C.S. Potten, The re-establishment of hypersensitive cells in the crypts of irradiated mouse intestine. Int. J. Radiat. Biol. 46 (1984) (in press).
3. K. Ijiri and C.S. Potten, Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation. Br. J. Cancer 47, 175-185 (1983).
4. A.H. Wyllie, Cell death: a new classification separating apoptosis from necrosis. In "Cell Death in Biology and Pathology" (I.D. Bowen and R.A. Lockshin, Eds.), pp. 9-34. Chapman & Hall, London, New York, 1981.
5. C.S. Potten and J.H. Hendry, Stem cells in murine small intestine. In "Stem Cells: Their Identification and Characterisation" (C.S. Potten, Ed.), pp. 155-199. Churchill Livingstone, Edinburgh, 1983.
6. H. Cheng and M. Bjerknes, The stem-cell zone of mouse small-intestinal epithelium. In "Cell Proliferation in the Gastrointestinal Tract" (D.R. Appleton, J.P. Sunter and A.J. Watson, Eds.), pp. 155-165. Pitman Medical, Turnbridge Wells, 1980.
7. J.H. Hendry and C.S. Potten, Intestinal cell radio-sensitivity: A comparison for cell death assayed by apoptosis or by a loss of clonogenicity. Int. J. Radiat. Biol. 42, 621-628 (1982).
8. C.S. Potten, C. Chadwick, K. Ijiri, S. Tsubouchi and W.R. Hanson, The recruitability and cell cycle state of some intestinal stem cells. Int. J. Cell Cloning 2, 126-140 (1984).
9. K. Ijiri, Cell death in mouse intestinal crypts induced by acute γ-ray irradiation or by continuous irradiation with γ-rays or β-rays. (Abstract, 2-C-13). J. Radiat. Res. 26 (1) (1985).

NIRS-M-52, pp 246 - 257 1985

Induction of Micronuclei and Some Other Abnormalities in Mouse Bone Marrow Following Tritium Expsoure

M.Kashima, H.Joshima and K.Fukutsu

Division of Radiation Hazards
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

The cytogenetic effects of tritiated water (HTO) and ^{137}Cs γ-rays were studied by micronucleus test with polychromatic erythrocytes. RFM/Nrs male mice aged 9-13 weeks were injected intravenously with HTO at 0.025, 0.05, 0.1 or 0.2 mCi/g or received total doses of 28-200 rad of ^{137}Cs γ-radiation in continuous exposure with a decreasing dose rate. Animals were sacrificed 2 and 3 days after exposure to HTO or γ-rays for the micronucleus test. Splenic weight and the number of femoral bone marrow nucleated cells were also measured. Absorbed doses to bone marrow from ^{3}H β-radiation were calculated.

The dose-effect curves of micronuclei induction were found to be almost linear in the groups irradiated with γ-rays.

However the curves for HTO were bending continuously upward with an initial slope higher than γ-rays. RBE values of ^{3}H β-rays relative to γ-rays for micronuclei induction were found to be approximately 2.2 and 2.7 for 2-day- and 3-day-exposure to HTO respectively. For splenic atrophy and reduction of the number of bone marrow nucleated cells, RBE values of 1.5 and 2.4 respectively were obtained for a 2-day-exposure to HTO.

INTRODUCTION

It is becoming important to evaluate the possible health hazards of tritiated water (HTO) or gas associated with increasing number of facilities where significant quantities of ^{3}H are handled. With respect to workers, rapid determination of radiation dose is required to assess the possible hazards. We are interested in "biological dosimetry" for evaluation of absorbed dose from incorporated tritium. The micronucleus test is a simple, rapid and sensitive method for determining chromosomal damage *in vivo* in hematopoietic tissues following exposures to mutagenic agents and radiation, and seems to be a method of choice. Therefore we studied cytogenetic effects in the bone marrow of the mouse by the micronucleus test after a single acute exposure to HTO and determined the RBE values of ^{3}H β-particles relative to ^{137}Cs γ-rays administered under defined conditions of dose and dose rate.

METHODS

A total of 150 RFM/Nrs male mice aged 9-13 weeks-old

weighing 29-34 g were used. They were injected intravenously with HTO at doses of 0.025, 0.05, 0.1 or 0.2 mCi/g body weight, or received total doses from 28 to 200 rad of ^{137}Cs-γ irradiation. The latter was done in continuous exposure with decreasing dose-rate simulated to HTO decay curve with a half time of 42 hours, by changing the position of mice day by day from a source of 10 Ci.

The micronucleus test (1) with polychromatic erythrocytes in the bone marrow was performed at 2 or 3 days after the commencement of exposure to HTO or γ-rays. Bone marrow cell suspensions were prepared by repeated pipetting from femoral shafts into TC199 medium + 10% fetal calf serum. Slide samples were prepared from diluted cell suspensions using cytospin apparatus (Tomy Seiko Ltd.). Splenic weight and the number of femoral bone marrow nucleated cells were also measured.

Preliminary experiments concerned with metabolism of tritium after injections of HTO at dose levels of 20 to 80 μCi/g were carried out using 47 mice for macroautoradiography and radiochemical assay by liquid scintillation counting.

Whole body autoradiography was carried out to observe the distribution pattern of volatile and non-volatile ^{3}H according to Ullberg's method (2). Each mouse was injected intravenously with HTO, 1 mCi per mouse. Frozen whole body sections of mice at 1 hour, 2 and 7 days after injection were exposed to Sakura MARG films at -20°C for 2 weeks. Autoradiograms of non-volatile ^{3}H were obtained by using the same section freeze dried.

The average absorbed doses to bone marrow were estimated for HTO from the energy emitted (average 0.0057 MeV), effective half life and specific activity of ^{3}H by using the well known formulae as follows:

The initial dose rate $D_i = 2.13\ \bar{E}C$ (rad/hr)

where $\bar{E}$ is average energy and C, the concentration of ^{3}H ($\mu Ci/g$). Then, the total dose at time t

$$D_t = \int_0^t D_i\ e^{-\lambda t}\ dt = (D_i/\lambda)(1 - e^{-\lambda t}) \quad \text{(rad)}$$

The specific activity (disintegration per g) of individual bone marrow samples was obtained by radiochemical analysis using solubilizer Soluene (Parckard Ltd.) and a liquid scintillation counter (Beckman LS7500). No specific attention was paid to the contribution of the non-volatile fraction to the total absorbed dose.

RESULTS

1. Metabolism and Dosimetry.

Whole body autoradiograms of the frozen and freeze-dried sections of mice 1 hour and 7 days after injection of 1 mCi HTO are shown in the Fig. 1.

Activities of ^{3}H in the frozen sample were distributed uniformly throughout the body except the compact bone and the contents of the gastro-intestinal tract which had relatively low concentration. Non-volatile ^{3}H was not detected autoradiographically in the 1 hour samples, but observed clearly in those at 1 week in tissues such as liver, kidneys, pancreas, G-I tract , salivary glands, brain, bone marrow, spleen, testes and hair follicles.

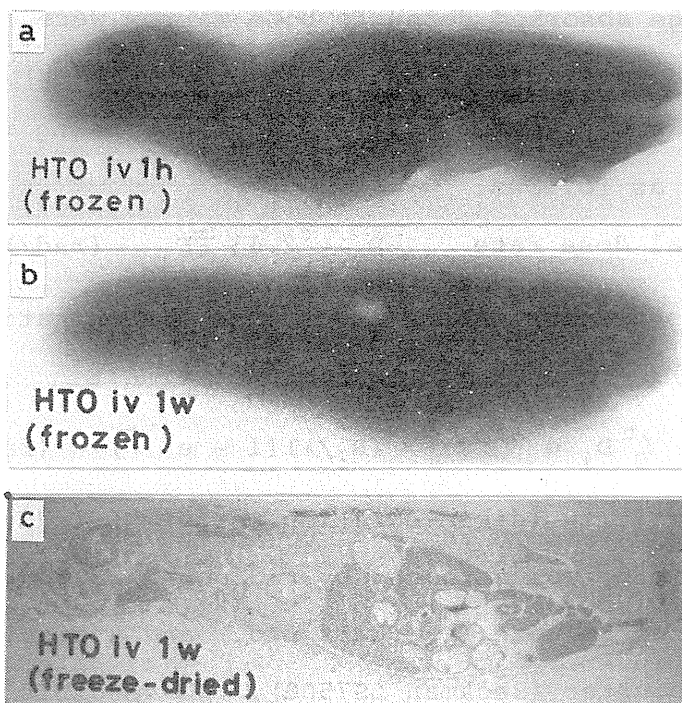


Fig. 1. Autoradiograms showing distribution of ^{3}H after a single injection of tritiated water in the mouse. (a) Distribution of ^{3}H in the frozen section 1 hour after injection. (b) Distribution of ^{3}H in the frozen section 1 week after injection. (c) Distribution of non-volatile ^{3}H 1 week after injection in freeze-dried section which is the same sample as above.

Water content of the several tissues was measured by weighing wet and dried samples with a semi-micro balance. The fraction of water (volatile fraction) obtained was as follows: 0.68 in bone marrow, 0.75 spleen, 0.81 testes, 0.68 liver and 0.74 lung. Although the specific ^{3}H activity was propotional to water content of the tissues, the biological half time of ^{3}H was not always the same among tissues having the same water content.

The ^{3}H activity in the bone marrow decreased exponentially with an average half time of about 42 (34-49) hours. The percentages of non-volatile ^{3}H at 2 hours and 3 days after

injection were 0.3 and 4.5 of the total activity in the bone marrow, respectively.

Variations with time of the activities in urine are shown in the Fig. 2. The half times of tritium in urine seem to be a little longer than those in the bone marrow and showed a tendency to extend with increasing injected dose of HTO. From all these considerations, the total average absorbed dose to bone marrow was calculated for HTO exposures and the continuous irradiations with ^{137}Cs γ-rays were designed to match the HTO exposures.

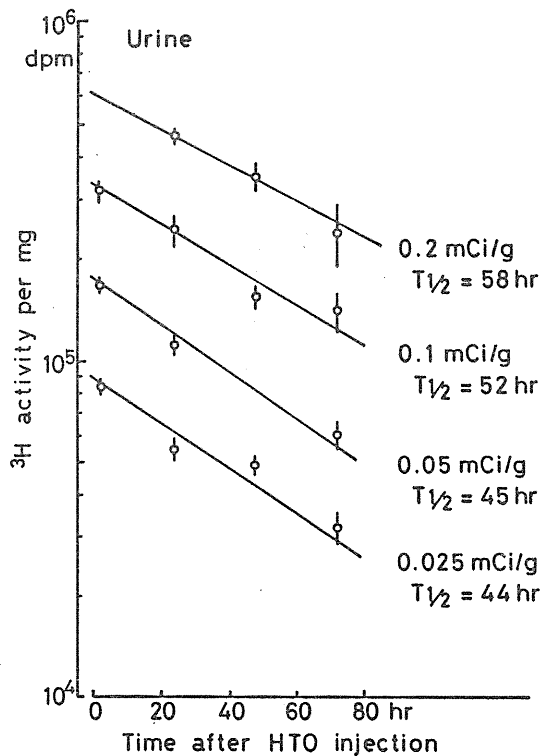


Fig. 2. Changes in tritium activity in urine after a single injection of tritiated water of 0.025, 0.05, 0.1 and 0.2 mCi/g body weight. $T^{1}/_{2}$ shows the biological half time.

2. Tritium Effects

Tables I and II summarize changes in the splenic weight

and the number of nucleated cells in femoral bone marrow and frequency of micronucleated polychromatic erythrocytes, after exposures to γ-rays and HTO at indicated doses and dose-rates.

It can be seen that the exposure to HTO produced effects about 2 times higher than that to ^{137}Cs γ-rays with all end

Table I

Summary of the effects of tritiated water and ^{137}Cs γ-rays 2 days after exposures in mice

Exposure level	Number of animals	Total BM dose (1st, 2nd day dose) rad	Splenic weight* (% of body weight) %	Number of nucleated BM cells in a femur* x 10^6	Rate of polychromatic erythrocytes with micronuclei %	net %	%/100 rad
Control	10	0	0.31 ± 0.02	17.3 ± 2.5	0.177(γ) 0.218(HTO)		
^{137}Cs-γ							
50 rad	5	50(30, 20)	0.28 ± 0.03	17.0 ± 3.0	0.769	0.592	1.18
100	5	100(59, 41)	0.25 ± 0.02	12.9 ± 2.3	0.906	0.729	0.73
200	5	200(118, 82)	0.21 ± 0.01	9.4 ± 0.6	1.266	1.089	0.55
HTO							
0.05 mCi/g	6	27	0.26 ± 0.02	15.0 ± 2.7	0.895	0.683	2.53
0.1	6	58	0.25 ± 0.01	12.1 ± 1.8	1.367	1.152	2.17
0.2	6	98	0.21 ± 0.02	9.2 ± 1.0	1.642	1.426	1.46

*mean ± S.D.

Table II

Summary of the effects of tritiated water and ^{137}Cs γ-rays 3 days after exposures in mice

Exposure level	Number of animals	Total BM dose (1st, 2nd & 3rd day dose) rad	Splenic weight* (% of body weight) %	Number of nucleated BM cells in a femur* x 10^6	Rate of polychromatic erythrocytes with micronuclei %	net %	%/100 rad
Control	9	0	0.34 ± 0.05	16.4 ± 2.4	0.196	0	
^{137}Cs-γ							
40 rad	5	40(19, 13, 8)	0.37 ± 0.07	15.1 ± 2.5	0.512	0.316	0.79
80	5	80(38, 25, 17)	0.31 ± 0.02	12.1 ± 1.4	0.861	0.665	0.83
150	5	150(71, 47, 32)	0.28 ± 0.01	10.0 ± 1.7	1.167	0.971	0.65
HTO							
0.025 mCi/g	6	16	0.28 ± 0.03	15.9 ± 2.9	0.591	0.395	2.47
0.05	4	31	0.25 ± 0.03	13.8 ± 1.2	0.831	0.635	2.05
0.1	9	68	0.24 ± 0.03	10.7 ± 2.0	1.094	0.898	1.32
0.2	7	129	0.20 ± 0.01	6.8 ± 1.7	1.431	1.235	0.96

*mean ± S.D.

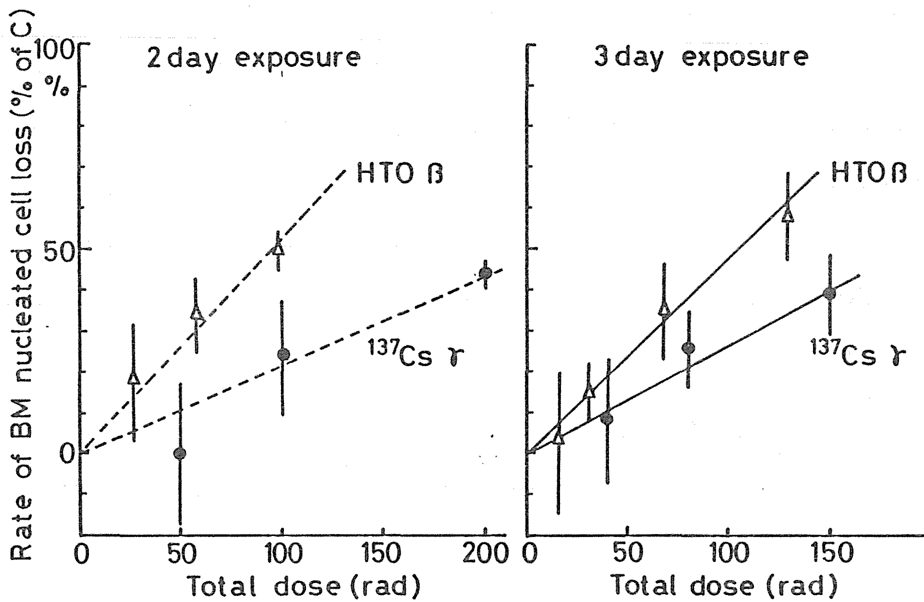


Fig. 3. Dose-response curves of the rate of femoral bone marrow nucleated cell loss for the controls and after 2-day- and 3-day-exposure to tritiated water or ^{137}Cs γ-rays.

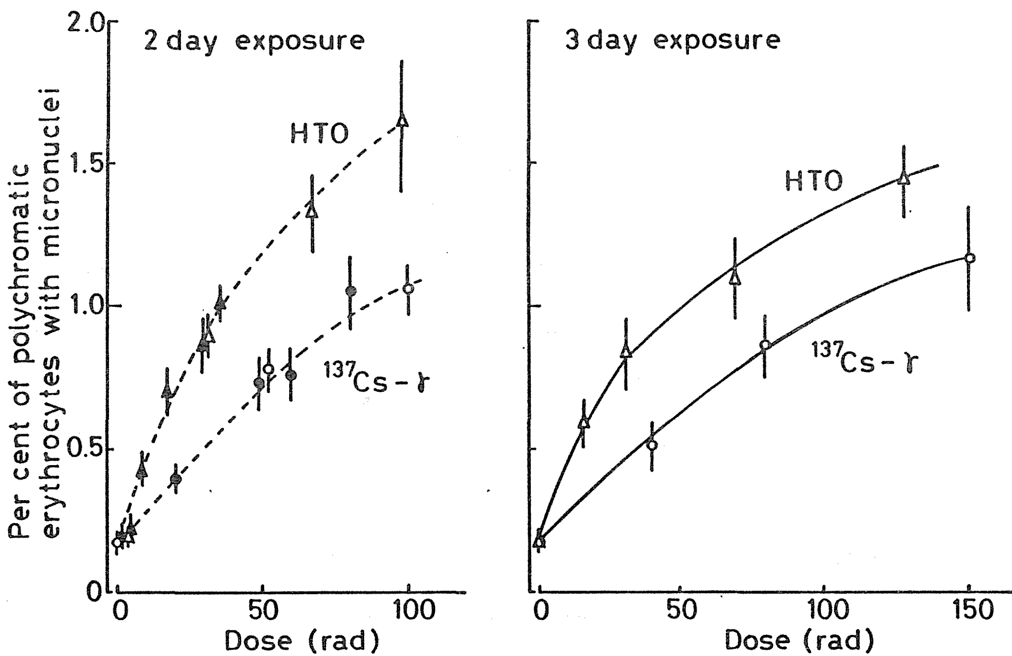


Fig. 4. Dose-response curves for micronuclei induction in the polychromatic erythrocytes of mice after 2-day- and 3-day-exposure to tritiated water or ^{137}Cs γ-rays.

points examined. Dose responses were analyzed and details will be shown in Figures 3-4 and in the next section. It should be pointed out that the net incidence of micronucleus formation for unit dose decreased with increasing total dose both for γ-rays and HTO. The changes after 2-day-exposure were found to be essentially similar to those after 3-day-exposure.

3. Estimation of Tritium RBE.

To estimate RBE values using ^{137}Cs γ-rays as the reference, dose-effect curves were constructed for splenic weight loss, bone marrow nucleated cell loss and micronucleus induction.

In the case of bone marrow nucleated cell loss the results shown in Fig. 3 could be fitted to linear equation as below:

2-day-exposure HTO : $Y = 0.54\ X$

γ : $Y = 0.22\ X$

3-day-exposure HTO : $Y = 0.5\ X$

γ : $Y = 0.267\ X$

Consequently, RBE of tritium β-rays were calculated to be 2.4 and 1.9 for 2-day- and 3-day-exposures, respectively.

Splenic weight loss:

2-day-exposure HTO : $Y = 7 + 0.156\ X$ (RBE 1.5)

γ : $Y = 4.4 + 0.14\ X$

3-day-exposure : adjusted equations were not obtained because of much individual variations in the values.

The RBE values for splenic weight loss were calculated to be 1.3-1.8 for 2-day-exposure. On the other hand, the curves for

micronucleus induction as shown in Fig. 4 were of continuously upward bending type, particularly for HTO. The reason for this is not understood well at the present time (The incidence after a single acute X-irradiation was linear to the dose). Therefore, the net increase of micronucleated polychromatic erythrocytes per unit dose was calculated for γ-rays and HTO exposures. Representative values are included in Tables I and II. From ratios of HTO relative to γ-rays at comparable doses and dose-rates, RBE values of HTO β-rays were calculated and found to be 2.2 and 2.7 for 2-day- and 3-day-exposure having a range from 2.0 to 3.0.

DISCUSSION AND CONCLUSION

Since HTO was observed by autoradiography to distribute almost homogeneously in the body of mice 1-7 days after injection, it was possible to calculate the average absorbed dose to bone marrow when the biological half time and specific activities are known.

The radiation doses from HTO estimated in the present experiments seem to be in accord with Furchner (3) who reported a value of 530-1650 rad for 0.7-1.41 mCi/g HTO for 30 days in the mouse.

When the splenic atrophy, decrease in the number of bone marrow cells and micronucleus induction in polychromatic erythrocytes were used as biological end points, RBE values approximately of 1.5, 2, 2.7, respectively, were obtained.

Storer et al. (4) reported values of 1.4, 1.6 and 1.7 for splenic and thymic atrophy (mice) and depression of ^{59}Fe uptake by red blood cells (rat), respectively for RBEs of

HTO relative to γ-rays. These values were obtained after an exposure for 5 days with HTO at dose range of 125-350 rad and seemed to be lower than those obtained in the present experiments. This may be due to the differences in the dose rates and times of measurement for each end point. For example a higher RBE was obtained for bone marrow nucleated cell loss after 2-day- than 3-day-exposure.

By using killing effect on mouse oocytes following *in utero* exposure, Dobson (5, 6) reported a maximum RBE of 3 for HTO (relative to γ-rays) at low dose rates.

Specific locus mutations in mice have been used as tritium-induced gene mutations and compared with those following X-ray-exposure. RBE values of 0.7 and 2.2 were reported for postspermatogonial and spermatogonial stage cells, respectively (7).

The RBE value for micronuclei induction in the mouse bone marrow seems to be high when compared to those obtained with other indicators, although it is in the range of previous reports.

REFERENCES

1. W. Schmid, The micronucleus test. Mutat. Res. 31, 9-15 (1975).
2. S. Ullberg, Autoradiographic studies on the distribution of labelled drugs in the body. Proc. Second U. N. Intern. Confer. Peaceful Uses Atomic Energy, 24, pp.248-254. 1958.
3. J. E. Furchner, Relative biological effectiveness of tritium beta-particles and Co^{60} gamma-rays measured by lethality in CF1 mice. Radiat. Res. 6, 483-490 (1957).
4. J. B. Storer, P. S. Harris, J. E. Furchner and W. H. Langham, The relative biological effectiveness of various ionizing radiation in mammalian systems. Radiat. Res. 6, 188-288 (1957).
5. R. L. Dobson and M. F. Cooper, Tritium toxicity: Effect of low-level ^{3}HOH exposure on developing female germ cells in the mouse. Radiat. Res. 58, 91-100 (1974).
6. R. L. Dobson, Low-level chronic exposure to tritium, an improved basis for hazard evaluation. In Biological and Environmental Effects of Low-Level Radiation, Vol. II, pp.57-63. IAEA, Vienna, 1976.
7. W. L. Russell, R. B. Cumming, E. M. Kelly and E. L. Phipps, Induction of specific-locus mutations in the mouse by tritiated water. In Behaviour of Tritium in the Environment, pp.489-497. IAEA, Vienna, 1979.

NIRS-M-52, pp 258 - 275 , 1985

Further Studies on the Genetic Damage to Bone Marrow and Other Somatic Effects Following Exposure to Low Level Tritium (^3H)

A.L.Carsten[1)], R.D.Benz[1)], S.L.Commerford[1,*)], W.Hughes[1)], Y.Ichimasa[2)], T.Ikushima[3)] and H.Tezuka[4)]

1) Brookhaven National Laboratory
Upton, New York 11973, U.S.A.

2) Department of Biology
Ibaraki University
Mito-shi 310, Japan

3) Research Reactor Institute
Kyoto University
Kumatori-cho, Sennan-gun, Osaka 590-04, Japan

4) Department of Induced Mutation
National Institute of Genetics
Yata 1, 111 Mishima-shi 411, Japan

ABSTRACT

This manuscript emphasizes results obtained over the last 3 years, supplementing information presented at the first ^3H workshop. Sister

---

*Deceased

Research supported by the U.S. Department of Energy under Contract No. DE-AC02-76CH00016 and under U.S.-Japan Fusion Cooperation Program, SA-53, DOE-STA

chromatid exchange (SCE) measurements on mice maintained on 3.0 μCi/ml of tritiated water (HTO) or receiving an equal depth dose ^{137}Cs gamma exposure for 52 weeks have been completed. Small but significantly higher numbers of SCEs were found in animals receiving gamma exposures or maintained on HTO than in their controls. In animals removed from the HTO regimen after 27 weeks, the number of SCEs decreases with time, but did not return to control levels within the first 30 weeks. Comparative studies with the ^{137}Cs exposures indicate an RBE not significantly different than 1.0. Mice were also maintained on 7.5, 15.0 and 30.0 μCi/ml HTO for SCE studies. Results similar to those described for 3.0 μCi/ml were found. Maintaining animals on a 0.15% saccharin solution is the most effective of those tested for increasing the animals' water intake and for enhancing excretion of ^{3}H. The rate of disappearance of ^{3}H from animals maintained chronically on HTO indicated a two phase ^{3}H disappearance curve from hemoglobin. Single injections of HTO showed a brief delay in incorporation of ^{3}H followed by a disappearance paralleling the slower phase of the chronic exposure curve, and indicating a RBC lifetime of approximately 40 days.

INTRODUCTION

The increased worldwide use of nuclear power reactors over the past several decades continues to stimulate interest in defining in greater detail the possible health hazards associated with their use. As shown by this second workshop in Chiba, Japan, there is particular interest in the possible hazards associated with tritium (^{3}H), a byproduct of fission energy generation and, to a significantly greater extent, a byproduct of fusion energy generation. Over the last several years a program has evolved in the Medical Department at Brookhaven National Laboratory to evaluate in mice some of the somatic, cytogenetic and genetic effects of acute and chronic tritiated water (HTO) ingestion or injection (Figure 1). Together with these studies, investigations into the metabolism of HTO have been undertaken. At the first of these workshops (1) and in several publications (2-15) details of earlier

studies have been presented. For the purpose of continuity, the results of these earlier studies will be briefly outlined here. Then the most recent studies on bone marrow cytogenetic effects, enhanced tritium excretion and determinations on the rate of incorportion and disappearance of tritium from the red blood cells (RBC's) of animals receiving either chronic ingestion or acute injections of HTO will be reported.

BROOKHAVEN TRITIUM TOXICITY PROGRAM

I. GENETIC AND REPRODUCTIVE EFFICIENCY
- A. Dominant Lethal Mutation Rate
- B. Cytogenetic Studies
- C. Examination of Ova and Early Embryos

II. SOMATIC EFFECTS
- A. Growth (Body Weight)
- B. Nonspecific Lifetime Shortening
- C. Bone Marrow Cellularity and CFU-S Content

III. RELATIVE BIOLOGICAL EFFECTIVENESS (RBE)
- A. Comparison of HTO and ^{137}Cs Effects

IV. BIOCHEMISTRY AND MICRODOSIMETRY STUDIES
- A. Rate of Tritium Incorporation
- B. Site of Tritium Incorporation
- C. Rate of Tritium Disappearance - Enhancement
- D. Histone and DNA Turnover Studies
- E. Cellular Turnover Studies

V. CARCINOGENESIS
- A. Induction of Leukemia

Fig. 1. Outline of Brookhaven Tritium Toxicity Program.

Summary of Earlier Studies

The results of all work on tritium toxicity (Tritox) performed in the Medical Department at Brookhaven National Laboratory are summarized in Table I. In earlier work, animals maintained chronically on 3.0 μCi/ml HTO through two generations have been found to appear normal grossly, grow at the same rate as their controls and suffer no significant lifetime shortening. Measurements on the incidence of dominant lethal mutation (DLM) induction showed that when both the male and female breeding partners are maintained on

3.0 μCi/ml, a significant reduction in viable embryos ($P<.0001$) and a significant increase in early deaths ($P<.01$) is observed. Similarly, when only the female is maintained on 3.0 μCi/ml, a significant reduction ($P<.01$) in viable embryos is seen. If both breeding partners are maintained on 1.0 μCi/ml, a significant ($P<.01$) reduction in viable embryos is noted. For all other measurements of DLM parameters for 3.0 and 1.0 or 0.3 μCi/ml, no significant effects are observed (early deaths, late deaths, corpora lutea).

Table I. Summary of results to date in the Brookhaven Tritium Toxicity Program.

TRITOX SUMMARY

	HTO CONCENTRATION (μCi/ml)					
	0.3	1.0	3.0	7.5	15.0	30.0
SOMATIC EFFECTS						
Growth	0	0	0	NA	NA	NA
Life-Time Shortening	0	0	0	NA	NA	NA
Bone Marrow Cellularity	0	0	0	NA	NA	NA
Bone Marrow CFU-S	0	+	++	NA	NA	NA
GENETIC EFFECTS						
Dominant Lethal Mutations	0?	+	++	NA	NA	NA
CYTOGENETIC EFFECTS						
Marrow Sister Chromatid Exchanges	0?	NA	+	+	+	+
Regenerating Liver Aberrations	0?	NA	+	NA	NA	NA
Micronuclei in Erythroid Cells	NA	NA	0	0	+	++

NA — Not Available.

Cytogenetic studies on the regenerating livers of animals maintained on 3.0 μCi/ml for 100, 330, 500 and 560 days exhibited a significant increase in the number of abnormal chromosomes in cells in the regenerating livers as compared to the control animals. Details of this study have been published (2). Similar effects were not found in animals maintained on 0.3 μCi/ml.

Measurements of micronuclei in red blood cells of mice maintained for 5-6 weeks beginning at 3 weeks of age on 3.0 to 30.0 μCi/ml HTO indicated a significant increase in micronuclei at the 30.0 μCi/ml level with a slight increase noted at 15.0 μCi/ml, but no evidence for an effect at 7.5 or

3.0 μCi/ml (R. Tice, R.D. Benz and A.L. Carsten, unpublished data).

No effect on the total cellularity of leg bone marrow was seen in any animals maintained chronically on 3.0 μCi/ml HTO or receiving equivalent external gamma ray exposures. In contrast, reductions in the number of bone marrow stem cells, as measured by the spleen colony technique (16), were noted as early as 8 to 10 weeks in the 3.0 μCi/ml mice and by 24 weeks in the 1.0 μCi/ml animals. In both groups, the stem cell depression continued with some variability throughout the lifetime of the animals. No effect was measurable in the 0.3 μCi/ml animals other than a somewhat greater than normal variability in the number of hemopoietic stem cells. Details of this study have been published (5,10).

Measurements of relative biological effectiveness (RBE) have indicated no significant difference ($P<.01$) between animals ingesting HTO and animals receiving equivalent external gamma ray exposures. However, for some of the parameters measured the effects were somewhat greater for the HTO, although not significantly so. This might be interpreted as an indication that for <u>those specific parameters</u> the RBE or Q value for HTO compared to external gamma ray exposure may be slightly greater than 1 but less than 2. In all cases the reference radiation used must be strictly defined since it is not difficult to find different RBE or Q values if a different reference radiation is used.

Studies on the biochemistry and microdosimetry of ^{3}H incorporation indicated that ^{3}H concentrations in body water and soft tissues rapidly approach equilibrium levels (3). When removed from a 3.0 μCi/ml HTO regimen, the ^{3}H level in tissue drops rapidly from 2.2 μCi/ml before withdrawal to 0.07, 0.01 and 0.001 μCi/ml at 7, 14 and 28 days later, respectively. The rate at which nonexchangeable ^{3}H disappears from brain and liver histones showed a half-life of 117 days for liver histone and 159 days for brain. Tritium activity in liver and brain indicated that the brain data points form a straight line with a slope indicating a half-life of 593 days contrasting

with the data for liver showing a pronounced curvature demonstrating the presence in the liver of two cell populations with distinctly different turnover times. These two liver cell populations exhibit half-lives of 12 and 318 days representing 23 and 77% of the total DNA, respectively. Further details of these studies have been published (6,8,10,11).

Current Studies

Results of investigations recently completed or still underway at Brookhaven primarily involved further quantification of the sister chromatid exchanges (SCE's) induced in mouse bone marrow by acute injections or chronic ingestion of HTO, metabolic studies and leukemogenesis studies. The results of the acute injection studies are being discussed in another presentation of this workshop by T. Ikushima. Current results of the chronic exposure studies carried out with both T. Ikushima and H. Tezuka, enhanced ^{3}H excretion studies carried out by Y. Ichimasa and studies on ^{3}H turnover in red blood cells carried out by W. Hughes will be discussed in the following paragraphs.

MATERIAL AND METHODS

Sister Chromatid Exchange Determinations and Cell Proliferation Kinetics

The details of the methods used for these evaluations have been recently published (14) and may be briefly described as follows: Random bred mice of the Hale-Stoner-Brookhaven strain were used for all determinations. Animals were weaned at 3 weeks of age and randomly divided into several groups. The experimental groups were maintained on drinking water containing HTO at concentrations ranging from 3.0 to 30.0 μCi/ml, while the control animals received tap water. All animals received food and water *ad libitum*. The food was Purina Rodent Laboratory Chow No. 5001, Ralston Purina Company, St. Louis, Missouri. HTO and control animals were housed in the same room on a 12 hour light and dark cycle. Temperature was maintained at 25°C ± 2° and at 50% ± 10% relative humidity. Animals were randomly selected from each of the groups at 1,2,4,8,12,18,22,27,32,36,44,52 and 60 weeks after being placed on the HTO regimen.

After those periods of time, animals were placed in a restraining apparatus and infused via the tail vein with 5-bromo-2'-deoxyuridine (BrdUrd, Sigma) at a rate of 50 mg/kg body weight per hour for 24-28 hours (17). Two hours before the end of the infusion, the animals were injected I.V. with colchicine at a concentration of 2 mg/kg body weight. Animals were sacrificed by cervical dislocation or CO_2 suffocation, the femurs removed and the bone marrow collected from one femur in each animal. Slides were prepared and stained as previously described (18,19). Twenty-five or 50 second division metaphases were examined from each animal. A minimum of 3 animals were used for each data point. Significance of differences between groups of animals was tested using analysis of variance tests using average square root transformed data. Cell proliferation kinetics were determined by counting the proportion of 100 metaphase cells that had replicated 1, 2 or 3 cycles in the BrdUrd environment.

Tritium Turnover in Red Blood Cells

For determinations in animals maintained chronically on 3.0 μCi/ml HTO, blood was obtained from animals which had been on the HTO regimen for periods of approximately 6 months and then placed on tap water. Samples were serially obtained at periods ranging from 0 to 80 days on tap water. The RBC's were separated by centrifugation and washed three times with 15 volumes of saline. They were hemolyzed in 25 volumes 0.01M, pH 7.7 phosphate buffer and then centrifuged at 18,000 rpm for 60 minutes. The solution was filtered through a 0.45 μm Millipore filter and the hemoglobin measured by optical density as Hgb-CN. A 5 ml aliquot was placed in a counting vial, freeze dried and redissolved in 0.15 ml 60% $HClO_4$ and an additional 0.3 ml 30% H_2O_2 added. Samples were stoppered and heated for 2 hours at 75°C to decolorize the solutions. To this sample was added 1 ml H_2O and 10 ml Scintiverse II (Fisher Scientific) for liquid scintillation counting. For determination of tritium in RBC's following single injections of HTO, the Hughes modification of the Anson and Mirsky globin technique (21) was used for separation of the

hemoglobin. Each animal received a 50 µCi i.p. injection of HTO which is nearly equivalent to the equilibrium dose in animals drinkings 3.0 µCi/ml of HTO. Blood was collected in hematocrit tubes at selected intervals from 3 to 54 days and centrifuged to obtain 30 to 60 mg of packed RBC's. These were resuspended in 0.1 ml H_2O precipitated with 1 ml of 0.1 N HCl in acetone and centrifuged in 1.5 ml microfuge tubes. The precipitate was washed once with 1 ml HCl-acetone and twice with 1 ml 100% CH_3OH. The precipitate at this time was white and dissolved readily in 0.5 ml H_2O. This solution was allowed to stand for 24 hours at 25°C to dissociate labile tritium from protein and then the material reprecipitated with 2 ml 0.1 M HCl in acetone and again washed 2 times with CH_3OH. The final precipitate was dissolved in 1 ml H_2O and transferred to a counting tube in which 10 ml Scintiverse II was added. The samples were then counted for ^3H activity.

Tritium Excretion Studies

All studies were done in a metabolism chamber designed and fabricated by Y. Ichimasa. This consisted of a cylindrical plastic container measuring 10 cm in diameter and 10 cm high. On the bottom of the container filter paper was placed and approximately 2 cm above the bottom there was a 1/4" wire mesh floor which allowed feces and urine to drop freely onto the paper below. The top of the cage was covered with a cylindrical mesh cover which allowed the insertion of the water bottle spout and a 10 ml plastic syringe in which a window was cut to serve as a food pellet dispenser. Immediately under the water bottle spout a small metal cup was placed to catch any water which might drip from the water bottle spout. (See Figure 2 for details). 100 male animals 3 weeks of age and of the same weight were selected from the mouse colony. One mouse was placed in each metabolism cage and the fluid measured by daily weighing of the water bottles and of the water in the collecting cup under the spout. The following solutions were tested to determine the mouse's preference on the basis of water consumption: 1) L-asparyl-L-phenylalanine methyl ester (Sigma), 2) cyclamic acid-sodium salt, (Sigma), 3) saccharin

(Sigma), and 4) tap water.

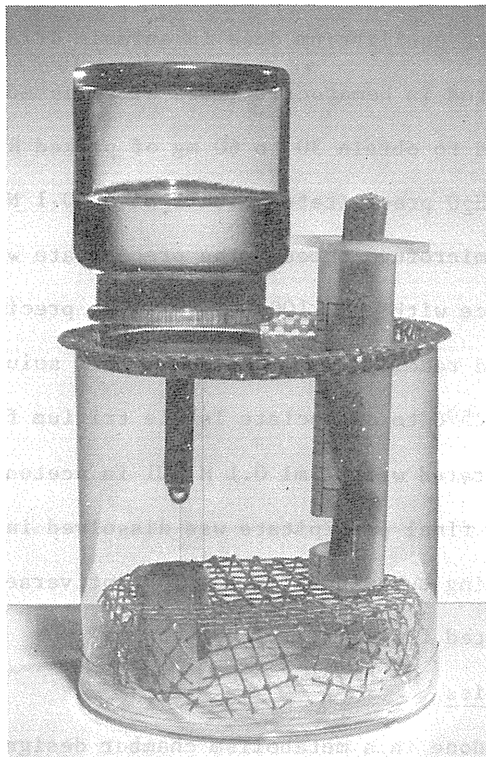


Fig. 2. Metabolism cage.

From these determinations it was found that the mice consumed the most liquid when the solution was 0.15% saccharin. On the basis of these results, further determinations were made to compare the effect of tap water or 0.15% saccharin solution available ad libitum on the reduction of body ^{3}H as measured by the ^{3}H content of the urine. The urine activity was determined by collecting 50 μl of freshly deposited urine on the paper on the bottom of the cage. This was then diluted with distilled water and the ^{3}H content determined by liquid scintillation counting.

Animal weights and hematocrit as well as food and water consumption were also measured throughout the experimental period.

Leukemogenesis Studies

A known effect of ionizing radiation exposure in mammals is the development of leukemia. This question is being investigated in mice of the CBA strain. This strain was chosen because it has a low incidence of spontaneous

acute myelocytic leukemia and a high incidence of the same disease after irradiation. Animals have received single whole body x-ray exposures of 50, 100, 200 or 300 rads (250 kVp, 100 rad per minute) at 3 and 9 months of age. In addition, other animals have received external whole body ^{137}Cs gamma exposures at dose rates of 1.2 or 1.8 rad per day (5 days/wk) until they have accumulated total doses of 300 rads. Additional animals receive fractioned (250 kVp x-ray) exposures (1-3 times/wk) to accumulated doses of 50-300 rads.

For comparison, equivalent aged animals have received either a single injection of HTO or continuous ingestion of 3.0 μCi/ml which would result in an integrated whole body dose equal to the x or γ exposures. All animals are examined daily for the first 6 months following exposure and twice daily thereafter to determine their health status. Sick animals which appear to be near death are sacrificed following peripheral blood counts (RBC, WBC and differential). Animals are then autopsied and microscopic evaluation made of liver, kidney, lung, spleen, mesenteric lymph nodes, femoral bone marrow, and sternum, including surrounding muscle. To date 220 mice have been injected with HTO and 225 with saline (controls), an equivalent number have received 250 kVp whole body x-ray exposures.

RESULTS

Sister Chromatid Exchange Evaluations

The induction of SCEs was examined in animals maintained chronically on doses from 3.0 to 30.0 μCi/ml for periods of up to 60 weeks. Animals were also exposed to ^{137}Cs gamma-rays at a dose equivalent to the 3.0 μCi/ml animals for RBE comparisons (Figures 3,4). In addition, animals were examined after various doses of single x-ray or HTO injections (work by Ikushima presented in this workshop). Results indicated that for all animals maintained chronically on all concentrations of HTO, the number of SCEs in bone marrow cells was higher in the HTO animals than in their comparable controls. The difference between control and exposed was small and increased with exposure duration. There was a very small, non-linear, dose response.

In animals removed from the HTO regimen and maintained on tap water, the difference between control animals and experimental reduced by approximately 50% within 10 weeks of removal from the HTO regimen but showed no further reduction by 30 weeks.

No significant effects on cell turnover rates were noted.

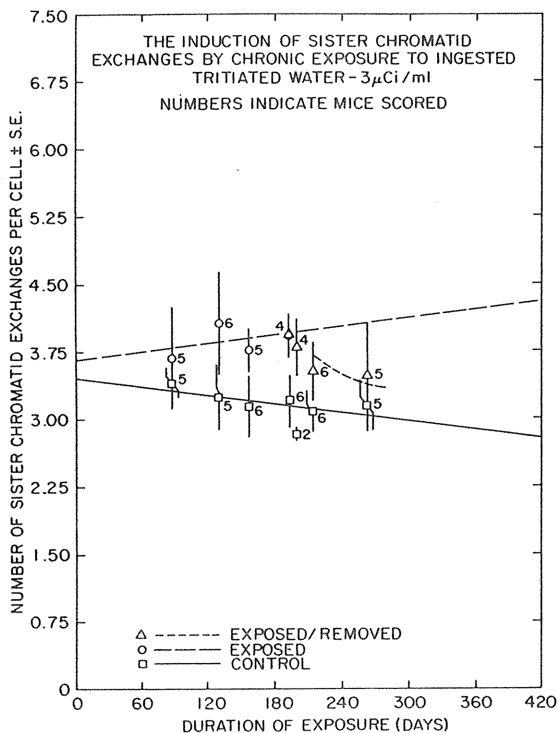


Fig. 3. The induction of sister chromatid exchanges by chronic exposure to ingested tritiated water - 3μCi/ml.

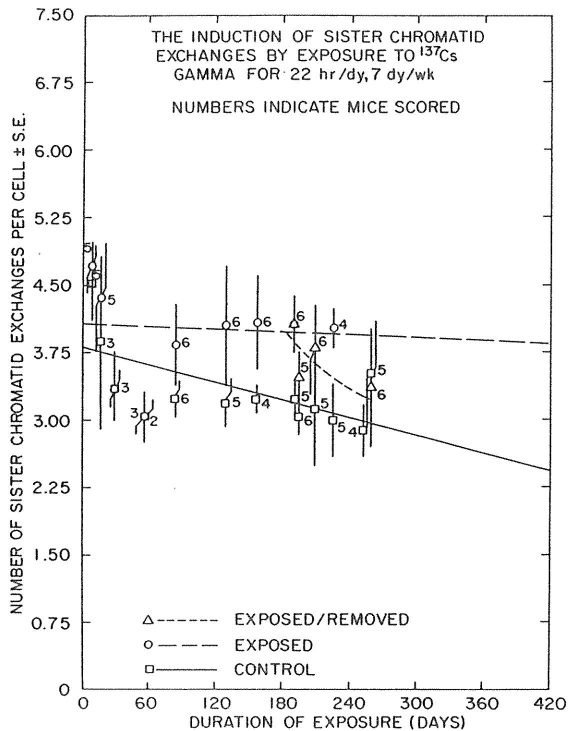


Fig. 4. The induction of sister chromatid exchanges by exposure to ^{137}Cs gamma for 22 hr/dy, 7dy/wk.

Tritium Turnover in Red Blood Cells

In the first study hemoglobin isolated from the red blood cells of mice maintained on 3.0 μCi/ml HTO for 26 weeks and then placed on tap water was followed for a period of 10 weeks. The disappearance curve for tritium from hemoglobin of the animals following chronic ingestion of HTO showed two components. The early phase, lasting for approximately 25 days, would extrapolate to zero at approximately 30 days. This is somewhat shorter than the expected life span of mouse erythrocytes (40 + days). The second portion of the curve extrapolated to slightly over 50 days. While this is slightly

longer than the expected life span, a correction should be made for the days required for labeled red cells to stop entering the vascular compartment.

In the second experiment with animals receiving a single exposure, the values for ^3H in hemoglobin showed no measurable incorporation for the first 3 days followed by a rapid rise reaching a peak at approximately 23 days. Following this, the curve fell rapidly to about 1/3 of the peak height and then reached a plateau which was followed by an abrupt drop to zero between 50 and 54 days, the same period as found in the first study for complete loss of label (Figure 5).

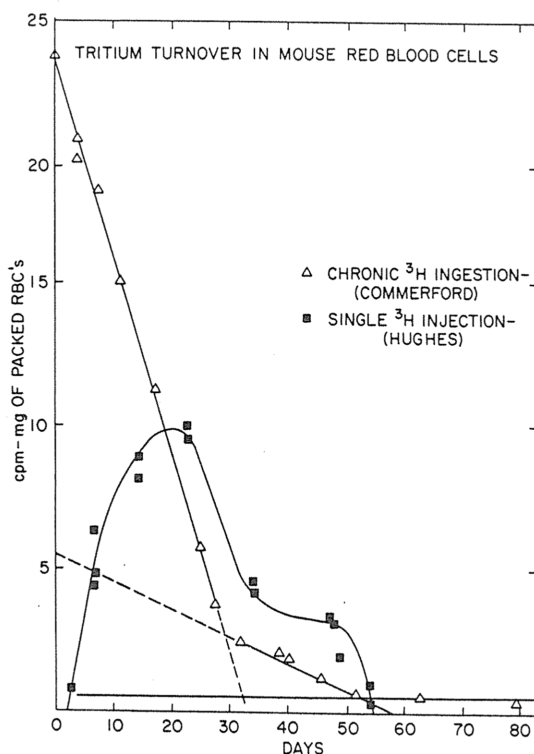


Fig. 5. Tritium turnover in mouse red blood cells.

Enhanced Excretion Studies

The results of the studies done by Y. Ichimasa indicated that the mice had a marked preference for a water solution of 0.15% saccharin. This difference was noted for both males and females. Measurements of the ^3H content in excreted urine over 27 days showed a marked increase in excretion for those maintained on the 0.15% saccharin solution. It was also noted that

the female mice excreted ^{3}H at a more rapid rate than did the males. (See Figures 6,7).

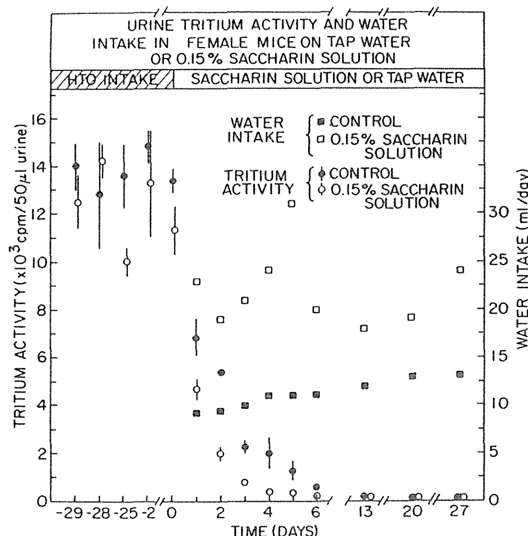


Fig. 6. Urine tritium activity and water intake in female mice on tap water or 0.15% saccharin solutions.

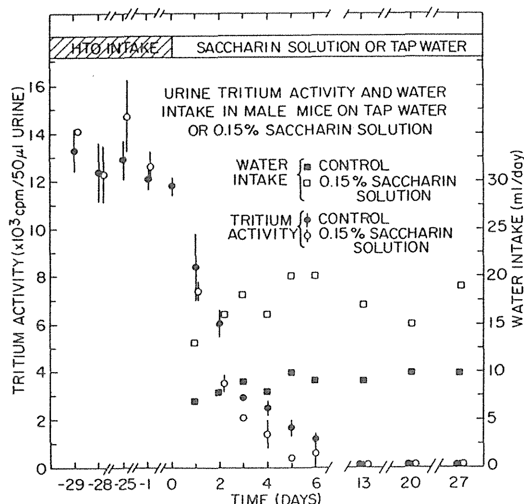


Fig. 7. Urine tritium activity and water intake in male mice on tap water or 0.15% saccharin solution.

Leukemogenesis Studies

As yet, too few animals have exhibited illness to make a definitive comment concerning whether or not the large single injections of ^{3}H induced leukemia or other malignancies.

DISCUSSION

Sister Chromatid Exchange

The results at all exposure levels indicate that SCEs are induced at low levels of ionizing radiation. At the same time, the lack of a linear dose response curve at relatively low doses may be taken as evidence of a saturation of the enzymatic pathway(s) by which ionizing radiation causes SCEs. The shape of the radiation induced SCE curve differs markedly from that for chemicals where essentially no saturation is found over several orders of magnitude. This can be seen in the work of Perry and Evans (18). Although they expressed their radiation dose response as a straight line, an examination of their data points shows a saturation effect not unlike that

which we have observed. These results indicate that there are at least two different molecular pathways that lead to SCEs.

Tritium Turnover in Red Blood Cells

The results observed in the turnover of ^3H in RBC's cannot be entirely explained by the known kinetics of red cell turnover. Mature red cells are known to survive for about 40 days in the blood stream of the mouse and then disappear as a cohort over the course of a few days. The results following a single pulse of HTO reported here show this behavior, except that the time for labeled cells to appear seems to be longer than expected and then the labeled cells do not maintain constant radioactivity throughout their life span. This suggests that much of their ^3H label is slowly exchangeable with a $T_{1/2}$ of several days. This postulate could also explain the results of the chronic experiment where 90% of the tritium disappeared quite rapidly, while the remainder slowly disappeared as though due to red cell death.

Enhanced Excretion Studies

The tritium excretion studies in animals drinking tap water and a variety of solutions containing artificial sweeteners indicate a preference by the animals for the 0.15% saccharin solution. As has been shown by previous investigators, the increased ingestion of fluid leads to a more rapid excretion of the tritium. The effect was more pronounced in the females.

CONCLUSION

In general, the overall results of the Tritox studies indicate that it is quite easy to detect genetic effects (DLM) and somatic (hematopoietic stem cells reduction) in animals maintained on HTO concentrations as low as 33 times the recommended maximum permissible concentration (MPC). Since other investigators have indicated evidence for an RBE or Q value somewhat higher than 1.0, this might lead one to question the suggested MPCs. However, a consideration of the practical situation arising from world-wide nuclear energy generation by either fission or fusion (7,20) makes it very clear that the world inventory for tritium would still be significantly below the MPCs.

However, this does not mean that one should ignore the possible hazards related to higher levels of tritium being present in the vicinity of reactors or reprocessing plants.

ACKNOWLEDGEMENTS

Over the past several years a number of scientists and technical assistants have been involved in this overall project. Many were acknowledged in the proceedings of the First Workshop on Tritium Radiobiology and Health Physics in Chiba, October 1981. Since that time, other individuals have taken active roles in this study and are hereby acknowledged. The authors wish to thank G. Hook, M. Nawrocky, and A. Mutschler for their excellent technical assistance. In addition, we wish to thank D. Pion and L. Wasson for their assistance in preparation of this manuscript.

REFERENCES

1. A.L. Carsten, A. Brooks, S.L. Commerford, and E.P. Cronkite, Genetic and Somatic Effects in Animals Maintained on Tritiated Water. Published in the Proceedings "Tritium Radiobiology and Health Physics," Workshop held at the National Institute of Radiological Sciences, Chibashi, Japan, NIRS-M-41, pp. 101-119, 1982.

2. A.L. Brooks, A.L. Carsten, D.K. Mead, J.C. Retherford, and L.R. Crain, The effect of continuous intake of tritiated water (HTO) on the liver chromosomes of mice. Radiat. Res. 68, 480-489 (1976).

3. A.L. Carsten, and S.L. Commerford, Dominant lethal mutations in mice resulting from chronic tritiated water (HTO) ingestion. Radiat. Res. 66, 609-614 (1976).

4. A.L. Carsten, and E.P. Cronkite, The genetic and hematopoietic effects of

long-term tritiated water (HTO) ingestion in mice. Pres. IAEC Symposium on Biological Effects of Low Level Radiation Pertinent to Protection of Man and His Environment, Chicago, Ill., Nov. 3-7, 1975, IAEA-SM-202/303, In: Biological and Environmental Effects of Low-Level Radiation, Vol. II, pp. 51-56, 1976.

5. A.L. Carsten, S.L. Commerford, and E.P. Cronkite, The genetic and late somatic effects of chronic tritium ingestion in mice. Current Topics in Ratiat. Res. Quat. 12, 212-224 (1977).

6. S.L. Commerford, A.L. Carsten, and E.P. Cronkite, The distribution of tritium in the glycogen, hemoglobin and chromatin of mice receiving tritium in their drinking water. Radiat. Res. 72, 333-342 (1977).

7. A.L. Carsten, Tritium in the Environment. In: Advances in Radiation Biology, Vol. 8, Academic Press, Inc., pp. 419-458, 1979.

8. A.L. Carsten, and E.P. Cronkite, Comparison of Late Effects of Single X-Ray Exposure, Chronic Tritiated Water Ingestion, and Chronic Cesium-137 Gamma Exposure in Mice. IAEA-SM-237/45, International Atomic Energy Agency, Vienna, pp. 269-276, 1979.

9. D.N. Slatkin, A.L. Carsten, S.L. Commerford, K.W. Jones, and H.W. Kraner, Genetic Hazard of ^3H: Estimation by Oocyte Uptake of ^2H. IAEA-SM-237/57, International Atomic Energy Agency, Vienna, pp. 231-240, 1979.

10. S.L. Commerford, A.L. Carsten, and E.P. Cronkite, Histone Turnover within Non-Proliferating Cells. Proceedings of the National Academy of Sciences 79, 1163-1165, 1982.

11. S.L. Commerford, A.L. Carsten, and E.P. Cronkite, The turnover of tritium in cell nuclei, chromatin, DNA and histone. Radiat. Res. 92, 521-529 (1982).

12. S.L. Commerford, A.L. Carsten, and E.P. Cronkite, The distribution of tritium among the amino acids of proteins obtained from mice exposed to tritiated water. Radiat. Res. 94, 151-155 (1983).

13. A.L. Carsten, "Tritox", a Multiple Parameter Evaluation of Tritium Toxicity. "Effects of Prenatal Irradiation" Symposium - XVIIth Annual Meeting, European Society for Radiation Biology, Bordeaux, France, July 26-29, 1982. Published in Proceedings.

14. T. Ikushima, R.D. Benz, and A.L. Carsten, Sister chromatid exchanges in bone marrow cells of mice maintained on tritiated water. Int. J. Radiat. Biol. 45, 251-256 (1984).

15. A.L. Carsten, The Tritium Toxicity Program in the Medical Department of Brookhaven National Laboratory. In: Annals of the New York Academy of Sciences, "Hematopoietic Cellular Proliferation", BNL, Upton, N.Y., in press 1984.

16. J.E. Till, and E.A. McCulloch, A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213-219 (1961).

17. E.L. Schneider et al., Methods in Cell Biology, Academic Press, New York, Vol. 20, p. 379 (1978).

18. P. Perry, and H.J. Evans. Cytological detection of mutagen-carcinogen

exposure by sister chromatid exchanges. Nature 258, 121-158 (1975).

19. P. Perry, and S. Wolf. New Giemsa Method for the differential staining of sister chromatids. Nature, 251, 156 (1974).

20. L.E. Feinendegen, E.P. Cronkite and V.P. Bond. Radiation problems in fusion energy predictions. Rad. Env. Biophys. 18:157-183, 1980.

21. M.L. Anson and A.E. Mirsky. The preparation of insoluble globin, soluble globin and heme. J. Genl. Physiol. 13:469-476, 1930.

NIRS-M-52, pp 276 - 286, 1985

Changes in the Hemopoietic Stem Cells and Lymphocyte Subsets in Humans after Exposure to Some Internal Emitters

I.Nakao, I.Jinnai, M.Bessho, Y.Kawase, M.Ohtani, H.Sugiyama and K.Hirashima[1)]

Division of Radiation Health
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260

1) Department of Internal Medicine
Saitama Medical College
Moroyama-machi, Saitama-ken 350-04, Japan

Summary

An attempt was made to evaluate the effect of radiation exposure on hemopoietic stem cells and cellular immunity in Bikini patients. Persons who had been administered Thorotrast were also studied.

No differences in the number of CFU-C between Bikini patients and normal controls were found at 30 years after exposure except some cases who received large doses, and no differences in the number of BFU-E, CFU-E and CFU-F between Bikini patients and controls were demonstrated. Marrow cells from Thorotrast cases have shown significantly decreased

numbers of CFU-F as compared with controls. Under this CFU-F depleted condition, the BFU-E and CFU-E values also decreased significantly. The CFU-C value in this CFU-F depleted marrow did not show significant differences from the controls. The CFU-F was considered to play a more important role than hemopoietic inductive microenvironment in erythropoiesis than in granulopoiesis.

Secondly, lymphocyte subpopulations in the above cases were analysed. In Bikini patients, the OKT 4 / OKT 8 ratio is increased in some cases. This imbalance in some cases may reflect immune senescence or an immune state that inclines to antibody production. In the Thorotrast cases, there is a decrease in the OKT 4 / OKT 8 ratio. The results may be regarded as a slight immunodeficiency state. Further, there is an increase in the percentages and the number of OKT 11^+,OKT 10^+, OKM1^+, OKIa-1^+ and HNK-1^+ lymphocytes. The results appear to reflect an increase in null cells or less differentiated T cells in periphral blood. It remains to be solved whether these changes depend on the mode and quality of radiation exposure.

Introduction

Medical follow-up of individuals who received mixed or internal exposure may provide valuable information regarding its radiation effects. In this workshop, our presentation appears to be not directly concerned with somatic effects of tritium. However, this study may have significance as a source of reference data for humans exposed to some internal emitters.

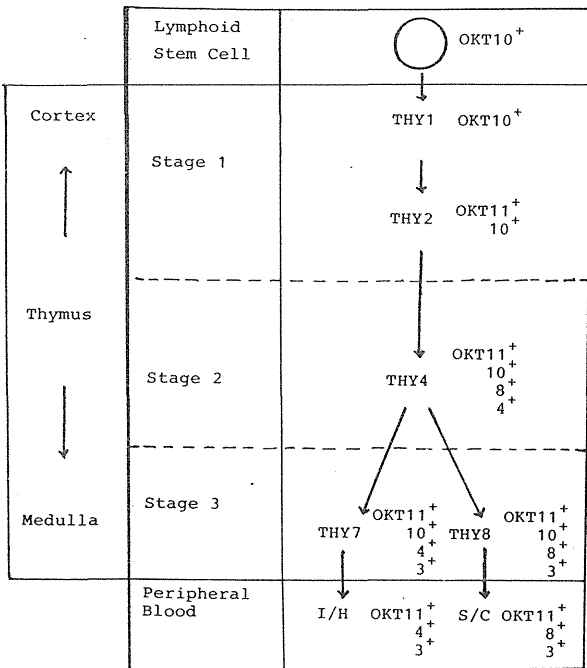
An attempt was made to evaluate the effects of radiation exposure on hemopoietic stem cells and cellular immunity in former fishmen exposed to radioactive fall-out at Bikini atoll. Persons who had been administered Thorotrast were also studied.

Materials and Methods

Stem cell analysis was made with bone marrow cells obtained from 5 patients who received mixed exposures caused by radioactive fall-out 30 years ago, and from 13 Thorotrast administered cases, who were injected with radioactive Thorium 232 dioxide (Thorotrast) for angiography over 30 years ago. In brief, buffy coat cells from marrow aspirate were collected. BFU-E (burst forming unit erythroid) and CFU-E (colony forming unit erythroid) were assayed following the method of Iscove[1] and scored at 7 and 14 days, respectively. CFU-C (colony forming unit in culture) was assayed following the method of Pike and Robinson[2] with some modifications. Cells were cultured for 14 days, and 10 % of GCT conditioned medium was used as CSF (colony stimulating factor). CFU-F (fibroblastoid colony forming unit) was assayed simultaneously. Briefly, 5×10^5 marrow cells were cultured with McCoy's 5A medium and 20% fetal calf serum, for 14 days. An aggregate consisting of more than 50 fibroblastoid cells was scored as a colony. Subpopulations of lymphocytes in peripheral blood were examined in 7 cases of Bikini patients, aged 48-54 years, and 11 cases of Thorotrast administered persons, aged 57-72 years, using several types of monoclonal antibodies. The antibodies including OKT 11, OKT 17, OKT 3, OKT 4, OKT 8, OKT 10, OKM1, OKIa-1 and OKB 7 were supplied by Ortho Diagnostic Systems Co.

Tokyo. HNK-1 (antihuman natural killer-1) monoclonal antibody was kindly provided by Dr. T. Abo, Tohoku University, Sendai.

The percentage of cells that expressed antigen reacting to a given monoclonal antibody was determined by flow-cytometry using Spectrum III. (Fig. 1,2)



I/H Inducer/Helper T cell

S/C Suppressor/ Cytotoxic T cell

Fig. 1. T lymphocyte differentiation and reactivity with monoclonal antibodies.

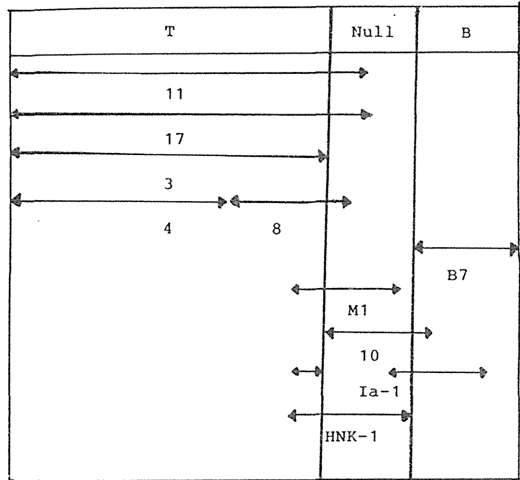


Fig. 2. Schematic outline of surface markers of human lymphocytes detected by monoclonal antibodies using in this study.

Results

No differences in the number of CFU-C between Bikini patients and normal controls were found at 30 years after exposure except for some persons who received large doses. Moreover, no differences in the number of BFU-E, CFU-E and CFU-F between Bikini patients and normal controls were demonstrated. On the contrary, marrow cells from Thorotrast administered cases have shown significantly decreased number of

CFU-F compared with normal controls. Under this CFU-F depleted condition in the marrow, the BFU-E and CFU-E values also decreased significantly compared with controls. On the other hand, the CFU-C value in this CFU-F depleted marrow did not show significant differences from the normal controls (Table 1). The results of Thorotrast cases are different from those of Bikini patients. They are regarded to represent injury of the stromal progenitor cells and erythroid precursor cells following the life long exposure to alpha rays from

TABLE I

COMPARISON OF QUANTITIES OF HEMOPOIETIC STEM-CELLS (PRECURSOR CELLS) BETWEEN UN-IRRADIATED CONTROL AND IRRADIATED PERSONS

Case	No. of Cases	CFU-F (No /5x10⁵ cells)	CFU-E (No/1x10⁵)	BFU-E (No/1x10⁵)	CFU-C (No/2x10⁵)
CONTROL	4	81.5 ± 33.3	89.8 ± 44.6	59.8 ± 17.9	109.3 ± 22.1
BIKINI	5	76.8 ± 27.8	91.6 ± 33.4	53.7 ± 9.7	68.2 ± 20.6
CONTROL	10	78.7 ± 28.7	90.9 ± 35.9	56.2 ± 13.0	76.1 ± 22.0
THOROTRAST	13	35.6 ± 24.4*	38.4 ± 22.9*	31.9 ± 10.3*	64.7 ± 34.2

* significant difference ($P < 0.001$)

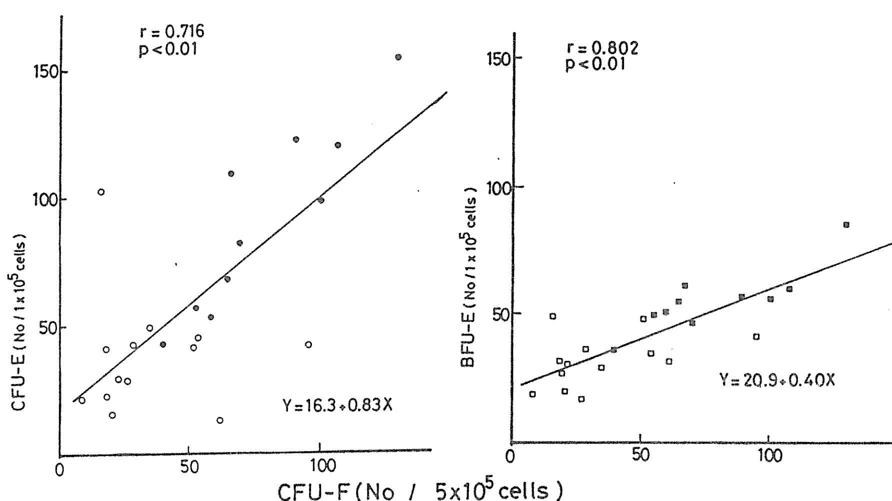


Fig. 3. CFU-F vs. erythroid precursors.

● ■ Normal subjects. ○ □ Thorotrast.

Thorotrast. As concerns the relationship between CFU-F and erythroid precursors, CFU-F values correlated well with those of BFU-E and CFU-E but did not with CFU-C (Fig.3). Therefore, CFU-F was considered to play a more important role than hemopoietic microenvironment in erythropoiesis than in granulopoiesis.

Secondly, lymphocyte subpopulations were analysed by flow-cytometry. In Bikini patients, there is an increase in the total number of OKT 3^+ and OKT 4^+ cells and decrease in the percentages of OKT 8^+ cells. The OKT 4 / OKT 8 ratio is increased in some cases.

On the contrary, in the Thorotrast administered cases, there is a decrease in the percentages of OKT 4^+ cells and in the OKT 4 / OKT 8 ratio. Further, there is an increase in the total number and percentages of OKT 11^+, OKT 17^+, OKT 10^+, $OKM1^+$,

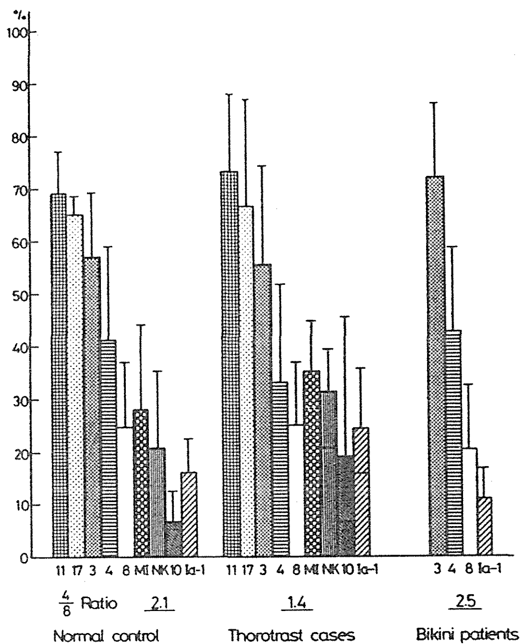


Fig. 4. Reactivity with monoclonal antibodies.

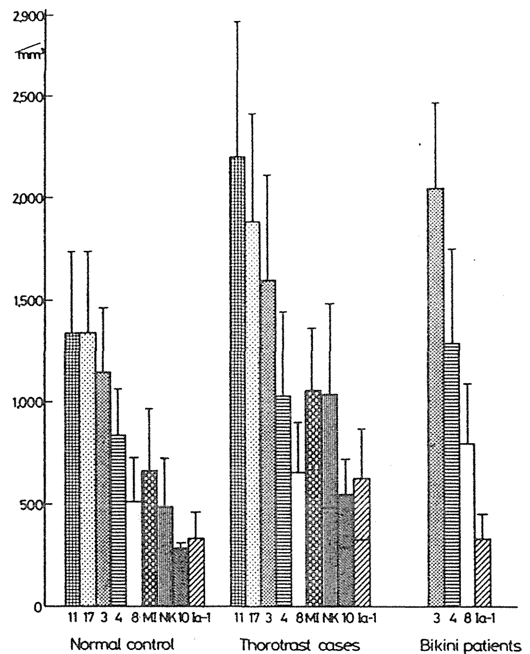


Fig. 5 Total number of lymphocyte subpopulations.

OKIa-1$^+$ and HNK-1$^+$ lymphocytes compared to age matched controls. The differences were statistically significant ($p < 0.05$). (Fig. 4,5)

The value for OKT 3$^+$ − (OKT 4$^+$ + OKT 8$^+$)(mm^3) was calculated by subtracting the sum of the number of cells reactive with OKT 4 and reactive with OKT 8 from the number of cells reactive with OKT 3. The difference between the number of OKT 3$^+$ cells and the sum of OKT 4$^+$ and OKT 8$^+$ cells showed more negative balance in Thorotrast cases ($196 \pm 153/mm^3$) compared to age matched controls ($123 \pm 125/mm^3$). (Data is not shown)

Discussion

Friedenstein et al. [3,4] have pointed out the possibility that fibroblastoid cells of marrow may constitute a major component of hemopoietic stroma and that CFU-F may be important to support normal hemopoiesis. Recently, it was reported that stromal precursors, represented by CFU-F, are sensitive to irradiation and the decrease of CFU-F in the marrow of irradiated mice continues for a long time.[5,6] The present work was undertaken to examine the possible injuries of bone marrow cells of irradiated persons. According to the results of simultaneous assays of stem cells in the marrow, the number of CFU-F was found to be correlated with those of erythroid precursors. Under the CFU-F depleted condition such as in Thorotrast administered cases, BFU-E and CFU-E values also decreased significantly but CFU-C did not show significant differences from the normal controls. CFU-F was considered to play a more important role than hemopoietic inductive microenvironment in erythro-

poiesis than in granulopoiesis.[7] Therefore, the examination of stem cells including CFU-F and erythroid precursor cells appears important for the evaluation of radiation hazard, because it is informative to the detection of the injuries in hematopoiesis.

Secondly, lymphocyte subpopulations in irradiated persons were analysed. In Bikini patients, the OKT 4 / OKT 8 ratio is increased in some cases. The OKT 4 / OKT 8 ratio represents a balance between helper-inducer subpopulation and cytotoxic-suppressor subpopulation of the peripheral T lymphocytes. An increase of the ratio indicates the increase of helper T cell activity relative to suppressor activity and suggests acceleration of cellular immunity frequently observed in aging humans [8,9] or in autoimmune diseases. This imbalance in some cases might be interpreted as immune senescence or an immune state that inclines to antibody production.

On the contrary, in the Thorotrast administered cases, there is a decrease in percentages of OKT 4^+ cells and in OKT 4 / OKT 8 ratio. The results may be regarded as a slight immunodeficiency state. Moreover, there is an increase in the number and percentages of OKT 11^+, OKT 17^+, OKT 10^+, $OKM1^+$, OKIa-$1^+$and and HNK-1^+ lymphocytes compared to age matched controls.

The OKT 11 and OKT 17 antigens are expressed by greater than 95% of thymocytes and are therefore considered to be early T cell antigens. In addition, about 50% of null cells (defined here as E^- , $SmIg^-$, OKT 3^- lymphocytes) bear the OKT 11 and OKT 17 antigens.[10]

The difference between the number of OKT 11^+or OKT 17^+ cells and OKT 3^+ cells (mature T cells) has been considered to

represent null cell subpopulation with natural killer cell activity. The OKM1 and natural killer antigens are expressed on most null cells, and OKT 10, a marker for human thymocytes, is also expressed on most null cells and lymphoid precursors.[10]

It was thought that an increased number of OKT 11^+, OKT 17^+, OKT 10^+, $OKM1^+$ and $HNK\text{-}1^+$ cells reflects an increased number of null cells. Further, a negative balance in the number of OKT 3^+ cells minus the sum of OKT 4^+ and OKT 8^+ cells is consistent with an increased number of less differentiated T cells in the peripheral blood,[11] because the thymocytes acquire OKT 4 and OKT 8 antigens before the expression the OKT 3 antigen on mature thymocytes.

The results appear to reflect an increase in null cells or less differentiated T cells in the peripheral blood. Albeit the functional significance of null cells in this cases is not yet fully understood, it seems likely that an increase of such cells reflects an increase in the number of natural killer cells.

It may be that a high level of null cells may represent a release of less differentiated T cells to the circulating blood as a homeostatic response to the perturbation of cellular immunity due to the life long internal exposure.

The major differences between Bikini patients and Thorotrast cases seems to be in the alteration of the ratio of helper and suppressor T cells and the behavior of null cells. It remains to be solved whether these changes depend on the mode and quality of radiation exposure.

References

1. Iscove, N.N., Sieber , F., Winterhalter, K.H.; Erythroid colony formation in culture of mouse and human bone marrow analysis of the requirement for eryhtropoetin by gel filtration and affinity chromatography on agarose-concanavalin A. J. Cell Physiol.,83, 309-320 (1974)
2. Pike, B.L., Robinson, W.A.; Human bone marrow colony growth in agar-gel. J. Cell Physiol., 76, 77-84 (1970)
3. Friedenstein, A.J., Deringlasova, U.F., Kulagina, N.N., Panasuk, A.K., Rudakowa, S.F., Luria, E.A.,Rudakowa, I.A.; Precursors for fibroblasts in different population of hematopoietic cells as detected by the in vitro colony assay method. Exp. Hematol., 2, 83-92 (1974)
4. Friedenstein, A.J., Chajakhjan, R.K., Latsnik, N.v., Panasyuk, A.F.,Keiliss-Borok, I.V.; Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Transplantation. 17, 331-340 (1974)
5. Friedenstein, A.J., Gorskaya, U.F., Kulagina, N.N.; Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp. Hematol., 4, 267-274 (1974)
6. Murate, T., Hotta, T., Utsumi, M., Yamada, H.; Murine marrow hemopoietic inductive microenvironment II. Radiation induced damages of stromal precursors and the function of their progenies analysed by a modified long term bone marrow culture. Acta Haematol. JPN. 47, 914-918 (1984)
7. Hirashima, K., Sugiyama, H., Jinnai, I., Bessho, M., Murohashi, I., Kawase, Y., Ohtani, M.; Study on the relationship between fibroblastoid colony-forming cells and

hemopoietic precursor cells using normal and CFU-F depleted human bone marrow. Exp. Hematol., (in press)

8. Moody, C.E., Innes, J.B., Staiano-Coico, L., Incefy, G.S., Thaler, H.T., Weksler, M.E.; Lymphocyte transformation induced by autologous cells XI. The effect of age on the autologous mixed lymphocyte reaction. Immunology. 44, 431-438 (1981)

9. Shwab, R.,Staiano_Coico, L., Weksler, M.E.; Immunological studies of aging. IX. Quantitative differences in T lymphocytes subsets in young and old individuals. Diagnostic Immunol. 1, 195-198 (1983)

10. Talle, M.A., Allegar, N., Makowski, M., Rao, P.E., Mittler, R.S., Goldstein, G.; Classification of human lymphocytes and monocytes with the OKT series of monoclonal antibodies. Diagnostic Immunol. 1, 129-135 (1983)

11. Hallgren, H.M., Jackola, D.R., Oleary, J.J.; Unusual pattern of surface marker expression on peripheral lymphocytes from aged humans suggestive of a population of less differentiated cells. J. Immunol. 131, 191-194 (1983)

NIRS-M-52, pp 287 - 295 , 1985

Background Tritium Levels throughout Japan

Y.Takashima

Department of Chemistry
Faculty of Science
Kyushu University
33 Hakozaki,Higashiku,Fukuoka 812, Japan

INTRODUCTION

Environmental tritium originates from several different sources, such as cosmic ray-induced reactions, nuclear explosions and atomic power plants. In developing the nuclear fusion energy, tritium will be the most important radioactive pollutant in the environment. Therefore, it is necessary to evaluate the present background levels of tritium in a variety of environmental samples before large scale tritium handling is under way. However, most tritium data reported in Japan are limited to natural waters in connection with nuclear installations.

We are measuring tritium concentrations for as many samples as possible in order to evaluate the present background levels throughout Japan.

MATERIALS AND METHODS

Secondary standard tritium solution of 65600 dpm/ml was prepared by diluting a standard solution purchased from Radiochemical Centre, Amersham. A commercially available scintillation cocktail, Aquasol-II (New England Nuclear Co. Ltd.) was used. Sixty milliliters of cocktail and 40 ml of water containing 1 ml of the standard tritium solution were mixed by shaking in a 100 ml Teflon vial. The samples thus prepared were kept in the counter for 4 days at 13°C. Then, the radioactivity and the external standard ratio were measured . An ALOKA low background liquid scintillation counter, LB-1, specially designed to meet the low background counting , was used[1)]. This system can be operated by using 100 ml Teflon counting vials. To eliminate the influence of an external standard source in sample measurement , the source is inserted by an user only when the external standard ratio is measured. Simultaneous separations of atmospheric H_2O(HTO), H_2(HT) and CH_4(CH_3T) were carried out by using the system as shown schematically in Fig. 1 .

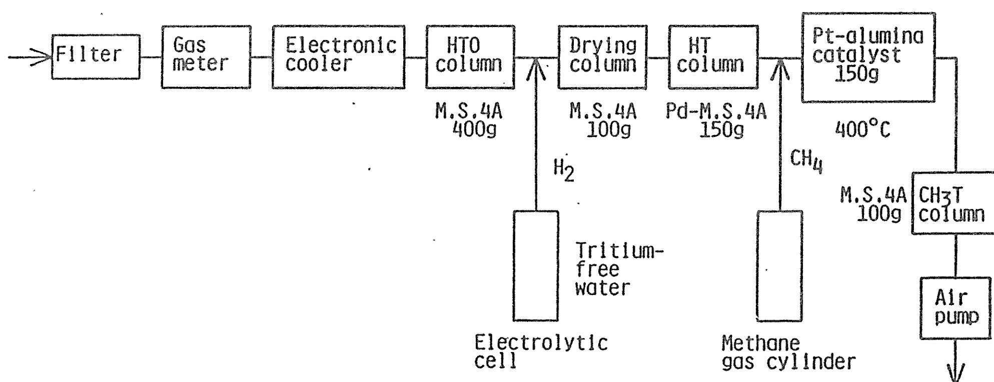


Fig. 1 Tritium sampler

This system was originally constructed at the Power Reactor & Nuclear Development Corp.[2], and partly modified by our laboratory to enable us to collect CH_4 in addition to H_2O and H_2.

About 4000 l of air is introduced into the system through the filter at a flow rate of 2 l/min. Water vapor is first collected in an electronic cooler and HTO column previously filled with 400 g of Molecular Sieve 4A. Dried air is then passed through the HT column which contains 150 g of molecular sieve carrying finely dispersed palladium metal. As the atmospheric hydrogen concentration is very low, some tritium-free hydrogen gas is added as a carrier. Thus, hydrogen is adsorbed in the HT column after catalytic oxidation to water. The remaining gas is finally introduced into the CH_3T column through the Pt-alumina catalyst bed, where methane is combusted. The resulting water is adsorbed into the CH_3T column. Tritium-free carrier gas is also used in this case. Water samples collected in a HTO column, a HT column and a CH_3T column are employed for liquid scintillation counting after distillation.

RESULTS AND DISCUSSION

Tritium Concentrations in Natural Waters

In order to know the recent environmental tritium levels in Japan, various environmental samples were collected from all over Japan in autumn, 1982 and 1983. The sampling locations are shown in Figs. 2 and 3. Twenty nine and twenty coastal seawater samples were collected in 1982 and in 1983, respectively. At each location, seawater was carefully sampled

at the seashore where no river flows into the sea to avoid the influence of river water. The lake waters were collected from 7 lakes in 1982 and 20 lakes in 1983. The initial 7 lakes are rather big and situated in Tohoku and Hokkaido area, while the latter 16 were located all over Japan. The river waters were collected only in 1982 from 5 rivers.

In addition to natural waters, pine needles were collected in 1983 from 21 points to compare the tritium levels in water extracted from them with those of natural waters.

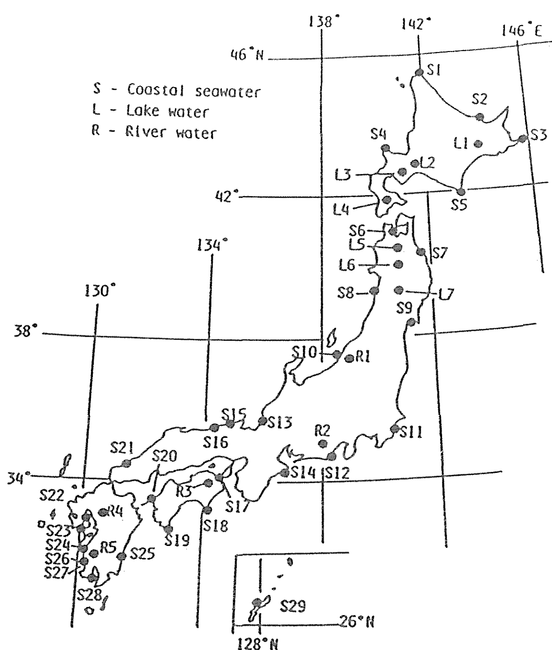


Fig. 2 Sampling locations in 1982

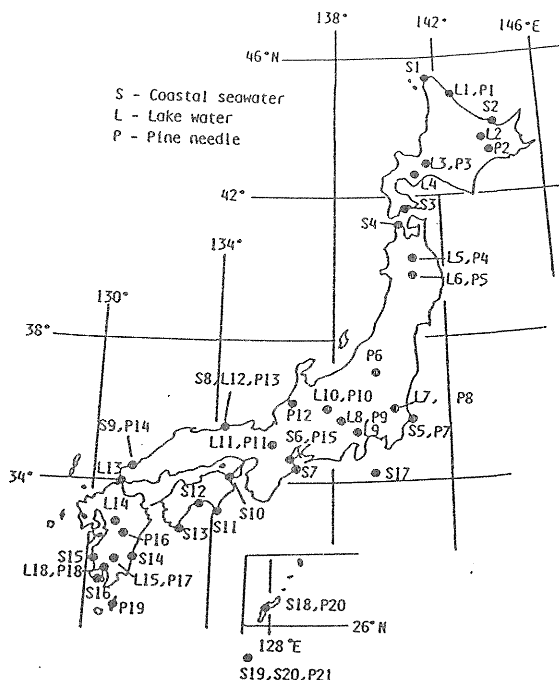


Fig. 3 Sampling locations in 1983

Tritium concentrations in coastal surface seawaters collected in 1982 and 1983 are summarized in Tables 1 and 2, respectively. Average tritium concentrations in coastal seawaters were 20.1 ± 3.5 pCi/l in 1982 and 19.3 ± 5.6 pCi/l in 1983. In spite of the different sampling locations, the average tritium concentrations between 1982 and 1983 are in

good agreement with each other. A value of about 20 pCi/l seems to be the background level of Japanese coastal seawater. This level is about two times higher than the recent eastern Pacific surface water level. This fact means that coastal seawaters are more or less affected by run-off from land.

Table 1. Tritium concentrations in coastal seawater in 1982

	Location	(pCi/L)
S-1	Cape Soya, Hokkaido	25.5 ±8.2
S-2	Abashiri, Hokkaido	22.2 ±6.4
S-3	Cape Nosappu, Hokkaido	25.5 ±7.8
S-4	Cape Kamui, Hokkaido	19.1 ±6.5
S-5	Cape Erimo, Hokkaido	21.0 ±7.4
S-6	Okunai, Aomori	21.2 ±5.7
S-7	Tanesashi, Aomori	15.1 ±7.9
S-8	Shimohama, Akita	15.9 ±8.4
S-9	Matsushima, Miyagi	21.2 ±7.5
S-10	Igarashihama, Niigata	18.6 ±6.1
S-11	Ohara, Chiba	17.0 ±7.3
S-12	Mochimune, Shizuoka	18.0 ±6.6
S-13	Tsuruga, Fukui	16.9 ±6.4
S-14	Kashikozima, Mie	22.9 ±8.5
S-15	Kasumi, Hyogo	21.5 ±7.0
S-16	Maruo, Tottori	25.0 ±7.7
S-17	Naruto, Tokushima	18.9 ±7.1
S-18	Cape Muroto, Kochi	19.0 ±7.0
S-19	Cape Ashizuri, Kochi	20.5 ±7.7
S-20	Nagasawa, Ehime	22.1 ±7.7
S-21	Hagi, Yamaguchi	19.0 ±7.8
S-22	Konagai, Nagasaki	26.3 ±8.1
S-23	Nagahama, Nagasaki	13.5 ±7.3
S-24	Nishikata, Kagashima	16.9 ±7.9
S-25	Nobeoka, Miyazaki	22.1 ±6.9
S-26	Sendai, Kagoshima	17.9 ±7.0
S-27	Yorita, Kagoshima	19.0 ±6.9
S-28	Kagoshima, Kagoshima	18.5 ±7.2
S-29	Onna, Okinawa	26.9 ±8.3
		av 20.1± 3.5

Table 2. Tritium concentrations in coastal seawater in 1983

	Location	(pCi/L)
S-1	Cape Noshappu, Hokkaido	29.4 ± 7.6
S-2	Abashiri, Hokkaido	21.4 ±7.5
S-3	Hakodate, Hokkaido	28.0 ±7.9
S-4	Cape Tappi, Aomori	20.2 ±7.2
S-5	Cape Inubo, Chiba	22.0 ±7.8
S-6	Toba, Mie	24.0 ±7.6
S-7	Futamiura, Mie	16.4 ±8.4
S-8	Nagaobana, Tottori	11.9 ±5.8
S-9	Nagato, Yamaguchi	12.4 ±6.7
S-10	Naruto, Tokushima	25.9 ±7.5
S-11	Cape Muroto, Kochi	16.2 ±7.9
S-12	Katsurahama, Kochi	16.6 ±7.4
S-13	Cape Ashizuri, Kochi	19.8 ±7.7
S-14	Totoro, Miyazaki	24.3 ±7.8
S-15	Sendai, Kagoshima	23.9 ±7.3
S-16	Nagasakibana, Kagoshima	21.8 ±7.6
S-17	Miyake Island, Tokyo	13.2 ±7.0
S-18	Inbu, Okinawa	11.6 ±5.8
S-19	Ishigaki Island, Okinawa	12.3 ±7.0
S-20	Taketomi Island, Okinawa	15.0 ±7.1
		av 19.3±5.6

Tritium concentrations in lake waters collected in 1982 and 1983 are summarized in Table 3. Tritium concentration of lake waters shows a great diversity from one lake to another. This can be attributed to their hydrological situations, especially to a residence time of water. Northerly located lakes, in general, have higher tritium concentrations than

those of southerly located lakes. The most reasonable reason for that is their size and depth, i.e., the northern lakes are generally larger and deeper than the southern lakes. The residence time of lake water is thought to be longer for the larger lakes. Average tritium concentrations for 7 lakes in 1982 and 16 lakes in 1983 are 99.3 ± 26.3 pCi/l and 63.5 ± 31.9 pCi/l, respectively. When we measured tritium in the lakes situated in Tohoku and Hokkaido, the average tritium concentrations in 1982 and 1983 were, however, almost the same.

Table 3 Tritium concentrations in lake waters in 1982 and in 1983

		Location	(pCi/L)
1982	L-1	Lake Akan	106.7 ± 8.4
	L-2	Lake Shikotsu	88.9 ± 7.9
	L-3	Lake Toya	144.3 ± 8.2
	L-4	Lake Onuma*	57.4 ± 7.8
	L-5	Lake Towada	87.3 ± 9.0
	L-6	Lake Onuma**	105.3 ± 8.8
	L-7	Lake Tazawa	105.1 ± 7.8
		av.	99.3 ± 26.3
1983	L-1	Lake Kutcharo	63.5 ± 7.3
	L-2	Lake Kussharo	113.5 ± 8.1
	L-3	Lake Shidotsu	76.5 ± 8.5
	L-4	Lake Toya	116.4 ± 8.1
	L-5	Lake Towada	117.2 ± 7.9
	L-6	Lake Tazawa	87.9 ± 7.9
	L-7	Kasumigaura	38.4 ± 7.0
	L-8	Lake Kawaguchi	53.7 ± 7.4
	L-9	Lake Yamanaka	68.6 ± 7.4
	L-10	Lake Suwa	71.2 ± 7.4
	L-11	Lake Biwa	51.9 ± 7.9
	L-12	Pond Koyama	36.8 ± 7.6
	L-13	Lake Toyoda	32.1 ± 7.1
	L-14	Lake Ezu	40.7 ± 7.0
	L-15	Pond Rokkannon	20.8 ± 7.0
	L-16	Lake Ikeda	26.8 ± 6.8
		av.	63.5 ± 31.9

* situated in Hokkaido
** situated in Akita Pref.

We also measured the depth profile of tritium concentration for one lake, the lake Shikotsu. As shown in Fig. 4 , no appreciable change in tritium concentration was seen with the depth.

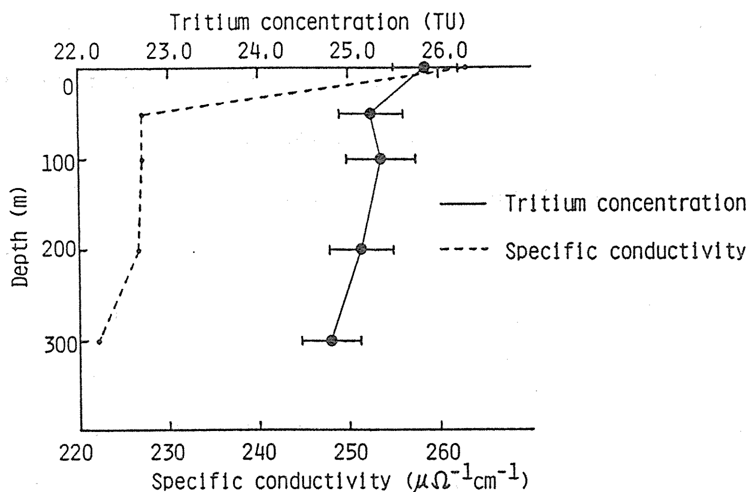


Fig. 4 Vertical profiles of tritium concentration and specific conductivity at sampling point 1.

Table 4 summarizes the average tritium concentrations of natural waters and pine needles measured in our laboratory. With respect to river water, only 5 samples collected in 1982 were used for tritium measurements. Average tritium concentration is higher than that of coastal seawaters but lower than that of lake waters. This means that river water has a shorter residence time than that of lake water.

Average tritium concentration of free water in pine needles collected from 21 locations in 1983 is 45.9 ± 14.8 pCi/l. In this case the concentration variation was not large, and no latitude dependence was observed. These values

Table 4. Average tritium concentrations in natural waters and pine needles

Sample	1982 (pci/L)	1983 (pci/L)
Coastal seawater	20.1 ± 3.5(29)*	19.3 ± 5.6(20)
Lake water	99.3 ± 26.3(7)	95.3 ± 18.7(6)
		63.5 ± 31.9(16)
River water	51.5 ± 11.8(5)	
Precipitation (in Fukuoka)	34.8 ± 20.6	31.5 ± 20.2
Pine needles		45.9 ± 14.8(21)

* The figures in parentheses represent the number of samples

are considered to represent the tritium levels of water in soil and in the atmosphere for the period before sampling.

Tritium Concentration in the Atmosphere

Tritium concentrations of different chemical species, HTO, HT and CH_3T, in the atmosphere in Fukuoka were measured from February 1983 up to the present. The results obtained are shown in Fig. 5. In cases of HT and CH_3T, no seasonal variation was observed with the values of 0.95 - 1.9 pCi/m^3 for HT and 0.21 - 0.75 pCi/m^3 for CH_3T. On the contrary, tritium concentration of HTO was greater in summer than in winter, with the value between 0.2 - 3.1 pCi/m^3. The same trend is seen in the HTO concentrations shown in the unit of pCi/l H_2O. Tritium measurements were conducted in Niigata[3] and Ibaraki[2], but only for HTO and HT, using almost the same method. The results obtained in Niigata and Ibaraki are basically in good agreement with our data both in HTO and HT.

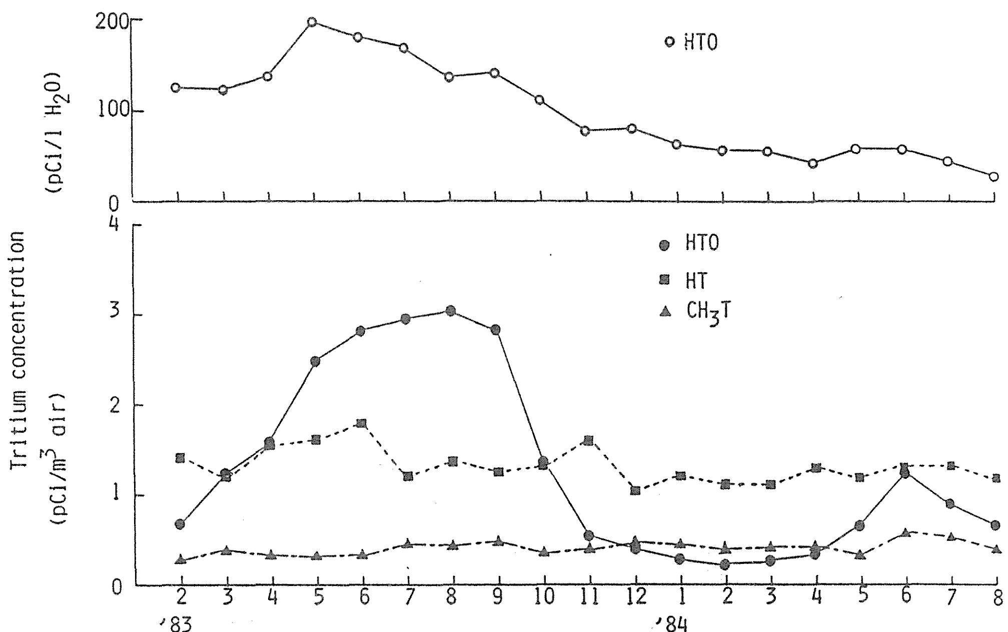


Fig. 5 Tritium concentration in air at Kyushu University

But several high HTO values, possibly due to nuclear installations, were observed in Ibaraki area.

The specific activities written in T.U. are about 30, 10^6 and 10^4 for HTO,HT and CH_3T, respectively. This fact obviously shows that the origins of tritium contained in these molecular species are quite different.

It is hoped that measurements of tritium concentrations in the atmosphere will be conducted at a number of places in Japan, because the available data is not sufficient to give the background levels in the atmosphere.

REFERENCES

1. T.Iwakura,Y.Kasida,Y.Inoue,and N.Tokunaga. A low background liquid scintillation counter for measuring low-level tritium. Proc.Symp., IAEA-SM232/32, 163-171(1979)
2. K.Shinohara,H.Watanabe,T.Nomura,A.Yamato, and M.Miura. Measurement and dose estimation of atmospheric tritium around the fuel reprocessing plant. Abstr.Symp.Atomic Energy Soc., Japan, 58(1983)
3. T.Hashimoto,Y.Higuchi,H.Takahashi and T.Sotobayashi. Measurement of HTO and HT and the construction of HTO electrolytic system. Abstr. 28th Symp. Radiochemistry, 235-236 (1984)

NIRS-M-52, pp 296 - 315 , 1985

Environmental Aspects of Tritium Released into the Atmosphere in the Vicinity of Nuclear Facilities in Japan

Y.Inoue, T.Iwakura and K.Miyamoto

Division of Environmental Health
National Institute of Radiological Sciences
9-1, Anagawa 4-chome, Chiba-shi 260, Japan

ABSTRACT

The environmental behaviour of tritium released into the atmosphere was studied in Tokai village at a residential area located between two nuclear facilities having tritium emission. Simultaneous sampling was carried out monthly for precipitation and ground water. The tritium concentration in the samples was determined by means of a low background liquid scintillation counter.

An inverse relationship was clearly observed between the monthly washout deposition of tritium and the source distance. The results agreed well with those calculated based on the washout deposition theory. A proportionality constant, important to estimate washout constant, was determined by comparing observed with calculated data. The excess tritium concentration in ground

water also decreased with the source distance and its origin was attributed to tritium released into the atmosphere from the two water moderated research reactors.

The washout deposition is suggested to be important to dose estimation for the people living in the vicinity of the emitters.

INTRODUCTION

To evaluate the radiological impact of tritium, better understanding of its environmental behavior is needed. When tritium is released into the atmosphere as vapor, its transport and distribution in the environment are strongly influenced by meteorological, geological and hydrological conditions. In addition to a direct pathway caused by atmospheric dispersion, it is also important to take into account an indirect pathway caused by washout deposition to the human body, because washout deposition is reported to be proportional to precipitation intensity and inversely proportional to a source distance (1). In Japan, we have much precipitation and a residential area is sometimes located close to nuclear facilities .

Since 1982 we have been carrying out a field study concerning the environmental behavior of tritium in the vicinity of nuclear facilities (2). The purpose of this work is to determine the relationship between tritium washout deposition and source distance along the downwind direction of certain nuclear facilities and compare it with that calculated based on a washout deposition model. The tritium concentration in the ground water was also determined in relation to the source distance.

MATERIALS AND METHODS

Site Description

Two nuclear establishments, having several tritium emitters at their sites are located along the coast at Tokai village, Ibaraki Prefecture in central Japan as depicted in Fig.1.

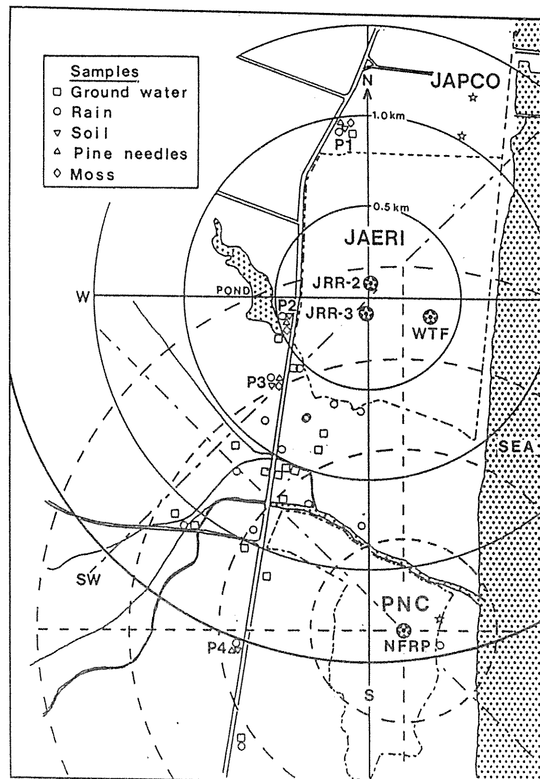


Fig.1: Sampling points for precipitation and ground water.

One is the Japan Atomic Energy Research Institute (JAERI) in which two heavy water moderated research reactors, JRR-2 and JRR-3, and a waste treatment facility constitute main tritium emitters. Another is the Power Reactor and Nuclear Fuel Development Corporation (PNC) with a nuclear fuel reprocessing plant (NFRP). Taking into account the meteolorogical condition of wind direction, population density and the availability of sample species, the sampling points are focused on the area between the JAERI and the PNC, especially the area located in the direction between south to southwest and at a distance of about 0.5 to 2.0km measured from JRR-2 and JRR-3.

Sampling and analysis

Samples of one month-precipitation and monthly colllected ground water were obtained at sampling points indicated in Fig.1. To evaluate baseline level, samples of each species were collected in regions far outside the map, and at Chiba city which is located at about 100km south from Tokai village.

Tritium concentration in water was determined by means of a low background liquid scintillation counter Aloka LB-1 in which fifteen 100ml Teflon vials can be loaded. Samples and blank water, 40 ml each, were mixed with 60ml of Aquasol-2 liquid scintillator (NEN) and counted for 500 minutes each. Electrolytic enrichment was applied to low level samples before counting, if necessary.

Source information, meteorological data and washout deposition model

Information on emission rates of tritium and meteorological data were obtained from periodicals published by the Ibaraki prefecture and also from publications of the JAERI.

The washout deposition of tritium was estimated based on the following equations (ref. 3), (see Appendix):

$$B_i = A \frac{N}{2\pi x} \sum q_{imt} \frac{\lambda_t}{U_m} \qquad \text{------ (1)}$$

where B = deposition per unit area ($Ci\ m^{-2}$)

A = activity emitted (Ci)

N = 16, number of sectors of wind direction

x = distance from emitter (m)

q_{imt} = frequency of precipitation,(i:sector of wind direction,

m:wind velocity level, t:precipitation intensity level)

U_m = wind velocity ($m\ s^{-1}$)

λ_t = washout constant (s^{-1})

and

$$\lambda = s\ \delta \qquad \text{------ (2)}$$

where s = proportionality constant ($a\ mm^{-1}s^{-1}$)

δ = precipitation intensity ($mm\ a^{-1}$)

<u>Assumptions for calculation of washout deposition of tritium</u>

Most sampling points have a potential to measure atmospheric tritium released from several emitters in the JAERI and the PNC. Through the year, the region is dominated by northern wind, especially by northwest wind in the winter and by northeast wind in the rest of the year. Since tritium washout deposition results from precipitation in the downwind direction of the emitters, higher washout deposition can be expected at the sampling points between south-southwest and southwest from the emitters. This is indicated by data on the frequencies of wind direction at precipitation and precipitation intensity in each wind direction shown in Fig.2. This situation prevails through the whole year according to the statistical data of the meteorology in this region (4). Therefore the area concerned seems to have a lower probability of the washout deposition for tritium released from the NFRP which is located in the south.

Figure 3 shows the rates of tritium emission into the atmosphere from main tritium emitters, such as, the JRR-2 and JRR-3 and the NFRP. The tritium emission rates of the NFRP were

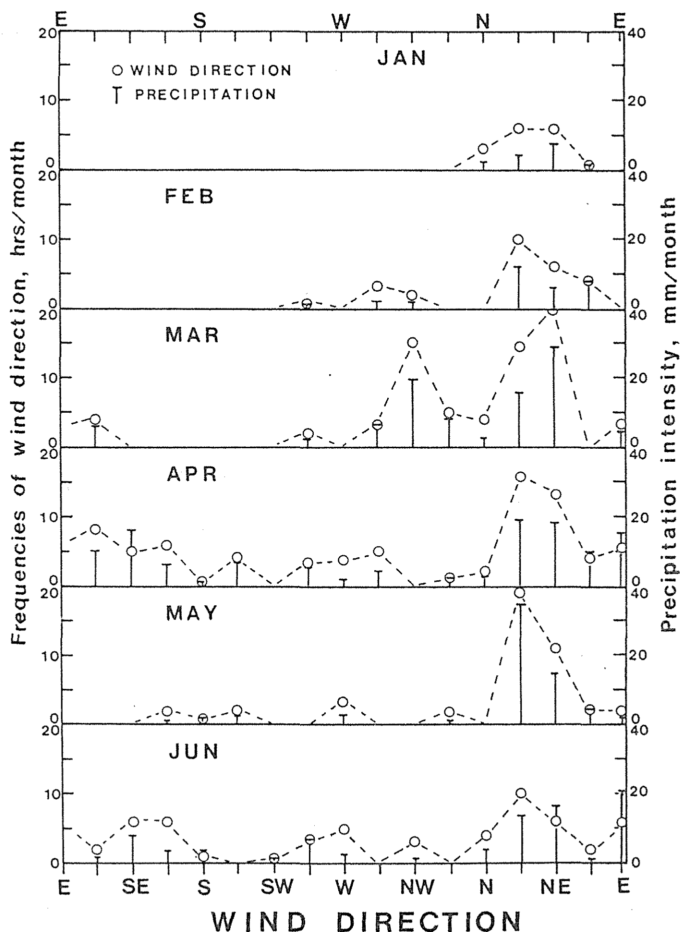


Fig.2: Frequencies of wind direction at precipitation and precipitation intensity in the first half of 1983.

usually several times higher than those of the JRR-2 and JRR-3. As the JRR-2 and JRR-3 are neighboring each other and release tritium continuously, they are regarded as a single emitter. Since the tritium released from the NFRP was composed of vapor and hydrogen the JRR-2 and JRR-3 may be regarded comparable to the NFRP in terms of emission rate in the vapor form. Other emitters released tritium at least several times smaller in amount than the JRR-2 and JRR-3 during the same period of months from 1981 to 1983 except December 1983.

Since the effective stack height is about 110m for the

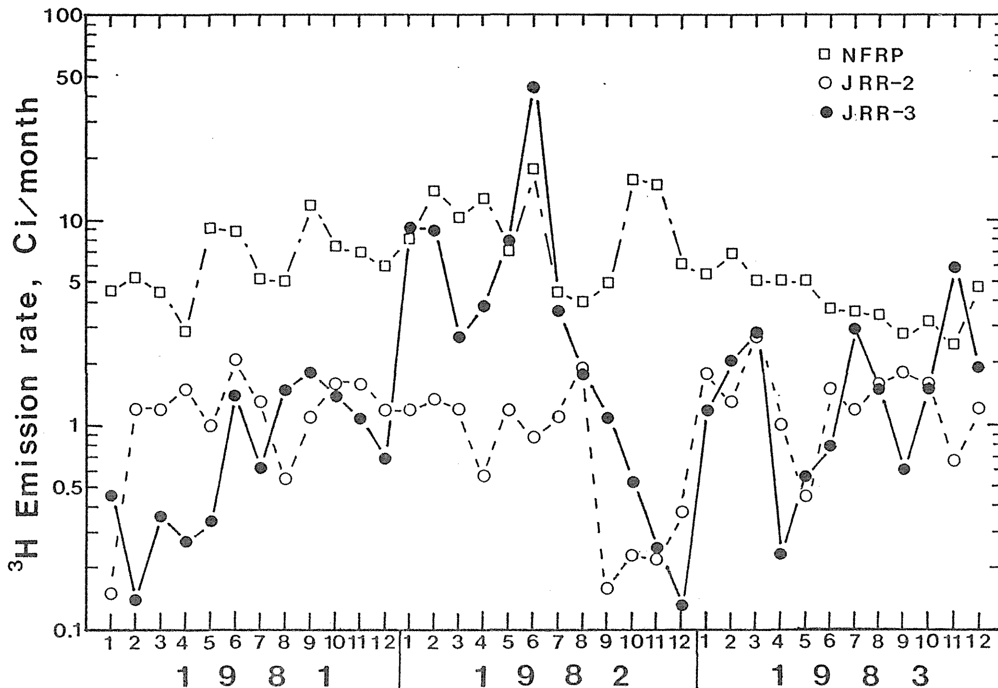


Fig.3: Tritium emission rates of major emitters from 1981 to 1983.

NFRP in the direction between WNW and N and about 50m for both the JRR-2 and JRR-3 in the direction between S and SW, the wind speed is considered to be about two times higher for the NFRP than those for the JRR-2 and JRR-3 at their effective stack heights. This fact suggests that tritium washout deposition is about two times less from the NFRP than from the JRR-2 and JRR-3 based on the equation (1).

Therefore, the contribution of the NFRP and other emitters can be ignored for calculation of washout deposition of tritium at the area concerned and only the contributions of the JRR-2 and JRR-3 will be taken into account as a first approximation.

It should be noted that a rather large amount of tritiated vapor was released from the JRR-3 in the middle of June 1982 followed by smaller amounts of tritium which are released from both the JRR-2 and JRR-3 in succeeding several months. This

incident may play an important role in the analysis of the environmental behavior of tritium in this area.

The following assumptions were made for calculation of washout deposition of tritium based on the above discussion:

1) No contribution of tritium from the NFRP, the WTF and other emitters except the JRR-2 and JRR-3.
2) The effective stack height is 50m for both the JRR-2 and JRR-3 in the downwind direction between S and SW.
3) The wind speed at 50m high is 2.6 times larger than that at 10m.
4) Uniform release rate of tritium during a month and uniform precipitation intensity in the area.
5) $s=2.6 \times 10^{-8}$ a $mm^{-1} s^{-1}$ as a parameter in the equation (2).

The third assumption is necessary because data of hourly wind speed were obtained only at 10m high at Muramatsu in the central part of Tokai village. They had to be converted to the ones at 50m high by applying the relationship between height

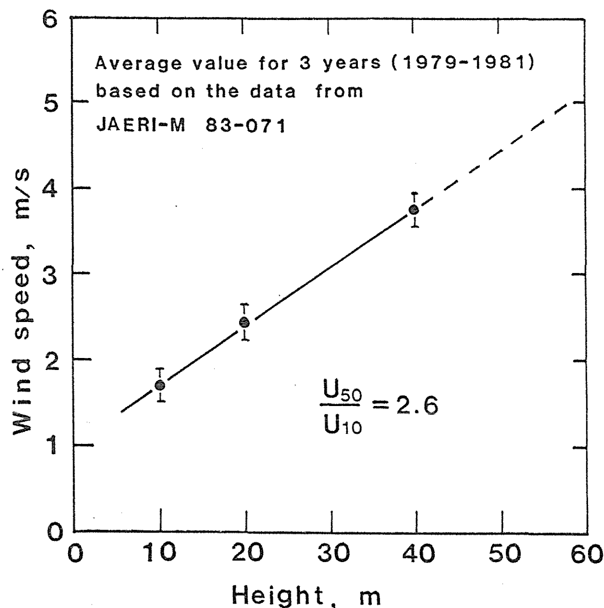


Fig.4: Relationship between height and wind speed.

above the ground and wind speed as shown in Fig.4 which was derived from the statistical data for the past three years (4).

RESULTS AND DISCUSSION

Observed and calculated monthly tritium deposition and tritium concentration in precipitation

The monthly tritium deposition and tritium concentration in precipitation were compared with those observed at the sampling point P3 from April 1982 to March 1983. The deposition was calculated according to the equations (1) and (2). Daily data concerning duration (hours) of precipitation, average wind speed ($m\ s^{-1}$) and average precipitation intensity ($mm\ h^{-1}$) converted to a unit of $mm\ a^{-1}$ were put in and summed up for a period of sampling, about one month only in case of northeast wind.

The tritium concentration of monthly precipitation ($nCi\ \ell^{-1}$) was calculated by dividing the washout deposition ($nCi\ m^{-2}$) by the amount of precipitation in the unit of mm, the amount of 1 mm being equivalent to an amount of water in ℓ per unit area, $1\ell\ m^{-2}$.

TABLE I

Observed and calculated monthly tritium deposition and tritium concentration in precipitation at P3

		1982				1983	
		APR	MAY	JUL	SEP	JAN	MAR
Deposition (nCi/m^2)	Obs.	6.4	32.1	67.5	11.2	3.9	17.9
	Cal.	11.8	72.5	41.1	8.0	2.4	29.9
RATIO (Obs/Cal)		0.54	0.44	1.6	1.4	1.7	0.60
Concentration (pCi/l)	Obs.	74	234	540	82	233	163
	Cal.	114	566	325	41	127	259
RATIO (Obs/Cal)		0.65	0.41	1.7	2.0	1.8	0.63

The results are shown in Table I for six selected months during 1982 to 1983.

Concerning the monthly washout deposition, the ratios of the observed to calculated values varied between 0.44 and 1.7, and the average with standard deviation was 1.0 $\pm$0.6. In the case of the tritium concentration in monthly precipitation, the ratios of the observed to calculated values varied between 0.41 and 2.0, and the average with standard deviation was 1.2 $\pm$0.7 as shown in Table I. It can be said from these results that the observed data agreed with the calculated ones within a factor of two, both for the monthly washout deposition and tritium concentration in precipitation, even though the several assumptions were used. This fact suggests that the proportionality constant of 2.6×10^{-8} a $mm^{-1}s^{-1}$ used in this work was quite reasonable and agreed with a value reported by Brenk, H.D. et al as shown in Table II.

TABLE II

Comparison of proportionality constants used for calculating washout constant

s (a $mm^{-1}s^{-1}$)	References	
3.4×10^{-8}	Brenk, H.D., et al.(1976)	(5)
3×10^{-9}	Hübschmann, W., et al.(1978)	(6)
$(1\text{-}2) \times 10^{-9}$	Papadopoulos, D. et al.(1982)	(3)
2.6×10^{-8}	This work (1984)	

The relationship between the washout deposition and the source distance

A typical example of tritium concentration in monthly precipitation around the JRR-2 and JRR-3 obtained in March 1983 is illustrated with bars on the area map in Fig. 5 in which numbers show the tritium concentration in the unit of pCi ℓ^{-1} observed at each sampling point A to H. The open part of each

bar corresponds to the natural level or fallout level observed in Chiba city and closed one to the excess tritium concentration that is the contribution of tritium from the emitters.

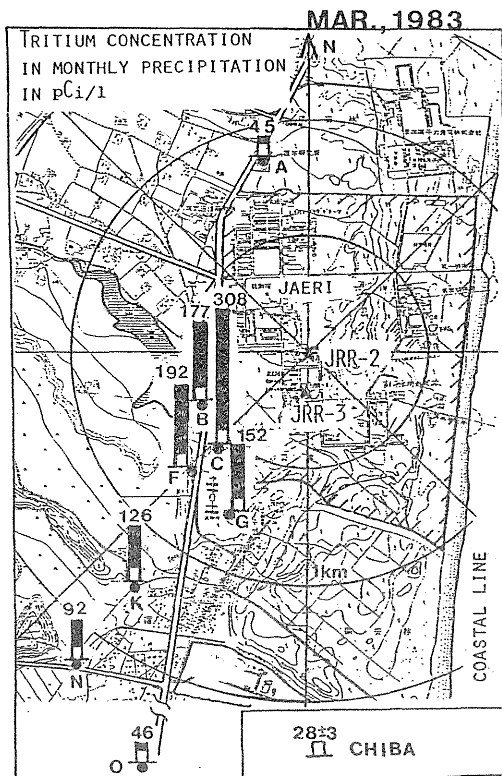


Fig.5: Tritium concentration in monthly precipitation observed along the south west direction of the JRR-2 and JRR-3 in March 1983.

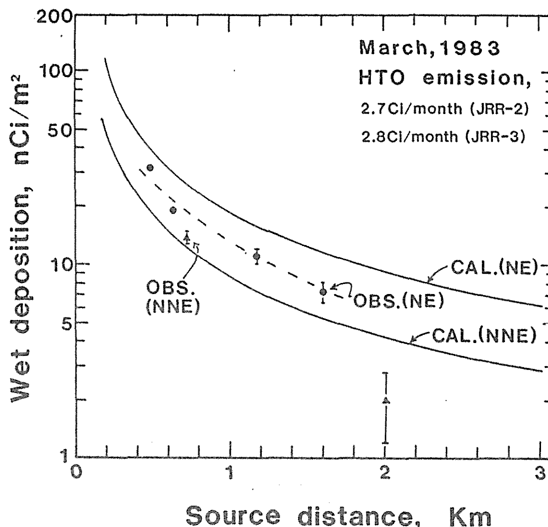


Fig.6: Relationship between washout deposition and distance from the JRR-2 and JRR-3 (comparison of observation and calculation)

It is clearly observed that the tritium concentration in precipitation decreased with the distance from the JRR-2 and JRR-3 in the direction of southwest. An attempt was made to simulate this situation by calculations based on the washout deposition model with the same assumptions as described above. The results are shown in Fig.6 in which the washout depositions were calculated for the wind directions both of northeast and north-northeast against the distance measured from the center of the JRR-2 and JRR-3. In the case of northeast wind, the

observed data obtained at the sampling points C,D,E and F were always lower than those calculated but agreed within a factor of two. In the case of north-northeast wind, two observed data available at the points G and H also agreed with calculations within a factor of two.

In conclusion, the washout deposition model, called sector model, used in this work proved to be valid and can be applied to predict both the washout deposition and tritium concentration in precipitation at any point.

The relationship between the tritium concentration in ground water and the souce distance

Once deposited the tritium can undergo one of several fates. The tritium can be transfered by infiltration through the soil and detected in the ground water. Figure 7(left) shows the relationships between the excess tritium concentration in monthly precipitation and the source distance in the southwest direction from the center of the JRR-2 and JRR-3 obtained for 6 successive months from January to June 1983. The excess tritium concentration in monthly precipitation decreased to one tenth with increasing source distance, from roughly 0.5 to 2.0km. The same concentration-distance relationship was observed in the excess tritium concentration in ground water samples collected on February 28 and June 6, 1983 along the southwest direction from the JRR-2 and JRR-3. The level in concentration was almost the same as that in the precipitaton during the same periods and decreased by one tenth from 0.5 to 2.0km as shown in Fig.7(right).

These facts suggest that the excess tritium in the ground water was the consequence of infiltration through the soil of

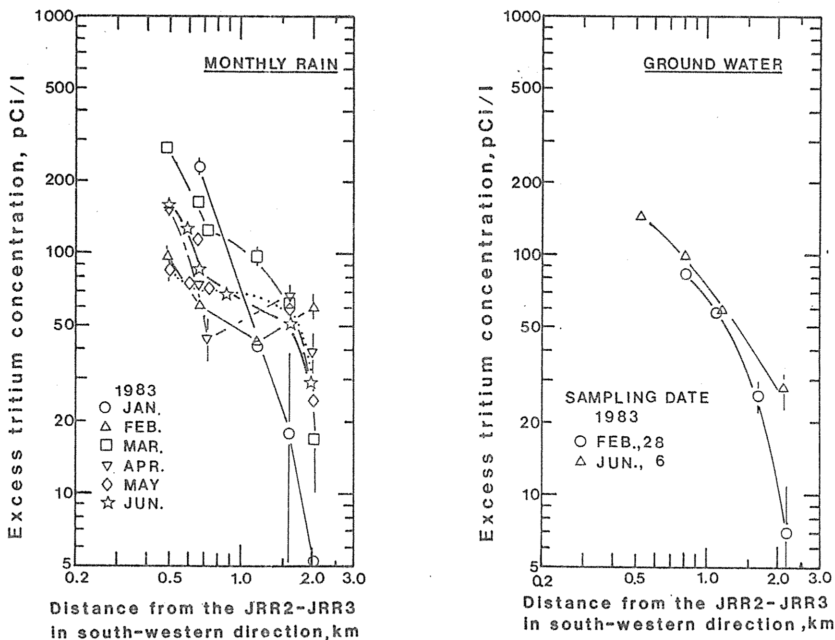


Fig.7: Comparison of monthly precipitation (left) with ground water (right) and the relationships between tritium concentration and source distance.

tritium deposited with precipitation at the surface of the ground in the area concerned before December 1982. A relatively large amount of tritium of 421 nCi m^{-2} was deposited in June 1982 compared with total amount of about 373 nCi m^{-2} accumulated for 23 months from January 1982 to December 1983. Only small amount of tritium was released in 1981 from the JAERI. In most of the area, a relatively fast velocity is expected in downward infiltration of water in the uppermost layers of soil which are composed of sandy soil up to at least 20m in depth. Therefore the excess tritium in the ground water could be attributed to the event in June 1982.

CONCLUSIONS

1) In the relationship between the monthly tritium washout deposition and the source distance, the measurerd values agreed

within a factor of two with the calculated ones based on the washout deposition model in which a proportionality constant was assumed to be 2.6×10^{-8}a $mm^{-1}s^{-1}$.

2) The area located between the JAERI and the PNC proved to be under the influence of tritium released into the atmosphere from the two heavy water moderated research reactors JRR-2 and JRR-3 in the JAERI.

3) The excess tritium concentration in the ground water decreased with increasing distance from the source almost in the same way as that in monthly precipitation in the area. Therefore, the excess tritium was considered to be introduced into the ground water as a consequence of the washout deposition of the tritium released from the JRR-2 and JRR-3.

ACKNOWLEDGEMENT

The authors are grateful to the Japan Atomic Energy Institute and the Ibaraki Prefecture for providing publications including meteorological data and information on tritium emission rates. This work is supported by a research grant from the Science and Technology Agency.

REFERENCES

1) K.J. Vogt, Models for assessing the environmental exposure by tritium released from nuclear installations. In Behaviour of tritium in the environment, pp.521-534, IAEA, Vienna (1979).

2) Y. Inoue, K. Tanaka and T. Iwakura, Ecological aspects of atmospheric discharged tritium in the vicinity of nuclear facilities in Japan. In RADIATION-RISK-PROTECTION, Compacts Volume 1. pp.180-183, Edited by A.Kaul et al., Fachverband

für Strahlenschutz e.V., Jülich (1984).

3) D. Papadopoulos, L.A. König, and K.-G. Langguth, Tritium contamination of rain water due to tritium release to the atmosphere. Annalse de l'Association Belge de Radioprotection, vol.7, no. 3-4. pp.129-145, (1982).

4) K. Obata, H. Kobayashi, H, Katagiri, H. Yabuta and M. Kokubu, Report of meteorological obervations on the site of Tokai Research Establishment from 1979 to 1981. JAERI-M83-071, JAERI (1983).

5) H.D. Brenk and K.J. Vogt, Konzeption für eine praxisnahe Berechnung der Ablagerung radioaktiver Stoffe aus der Abluft kerntechnischer Anlagen durch Niedersclag, Jül-1328 (1976).

6) W. Hübschmann and D. Nagel, ISOLAIII-Ein FORTRAN IV-Programm zur Berechnung der langfristigen Dosisverteilung in der Umgebung kerntechnischer Anlagen, Kfk-2698, Kernforschungszentrum Karlsruhe (1978).

APPENDIX

The most widely used model of diffusion is the continuous point source Gaussian plume formula:

$$\chi(x,y,z) = Q(2\pi\sigma_y\sigma_z u)^{-1}\exp(-y^2/2\sigma_y^2)[\exp(-(z-h_e)^2/2\sigma_z^2) + \exp(-(z+h_e)^2/2\sigma_z^2)] \quad (1)$$

where

$\chi(x,y,z)$ = steady-state concentration(Ci/m^3) at a point(x,y,z)

Q = the uniform emission rate of a radionuclide(Ci/s)

u = the mean wind speed in x-direction(m/s)

σ_y, σ_z = horizontal and vertical standard deviation of concentration distribution(m)

h_e = effective source height(m)

Values for σ_y and σ_z can be obtained from Pasquill-Gifford curves as a function of downwind distances. For concentrations calculated at ground level, i.e., z=0, the equation can be simplified to :

$$\chi(x,y,0) = Q(\pi\sigma_y\sigma_z u)^{-1}\exp(-y^2/2\sigma_y^2)\exp(-h_e^2/2\sigma_z^2) \qquad (2)$$

These equations are, however, valid when averaged over a time of one hour because such a time period smooths the effect of the turbulent fluctuations and gives a mean value that is quasi-steady. Over a longer period of time such as months or a year, the horizontal cross-wind distribution in the plume, represented by σ_y, will no longer be important since the wind direction frequency distribution becomes fairly uniform. In this case, the sector model can be used, where it is assumed that there is no horizontal variation in concentration within an angular sector equal to the resolution of the wind direction data. If the wind directions are taken at N points and it is assumed that the effluent is uniformly distributed in the horizontal within the sector and that the concentration does not drop off exponentially from a definable center line, the conception of the cross-wind integrated concentration (χ_{CWI}), which is equivalent to an average cross-wind concentration at a particular downwind distance(x) along a cross-wind line($2\pi x/N$), can be introduced. The value is obtained from the equation(2) integrated with respect to y from $-\infty$ to $+\infty$ giving:

$$\chi_{CWI} = 2(2\pi)^{-\frac{1}{2}}Q(\sigma_z u)^{-1}\exp(-h_e^2/2\sigma_z^2) \qquad (3)$$

Substituting the equation(3), the relationship

$$\chi_{AVG}(2\pi x/N) = \chi_{CWI}$$

reduces to the formula for ground level concentration within a sector i of arbitrary angle with $2\pi/N$ in radian:

$$\chi_{AVG} = NfQ(2\pi^3)^{-\frac{1}{2}}(\sigma_z ux)^{-1}\exp(-h_e^2/2\sigma_z^2) \qquad (4)$$

where f is the fraction of the time the wind blows toward that sector. The formula is sometimes modified to obtain the long term average concentration by summing all the concentrations and weighting each one according to its frequency for a particular stability category j and wind speed class m as follows

$$\chi_{AVG} = NfQ(2\pi^3)^{-\frac{1}{2}}(x)^{-1}\sum_j(\sigma_{zj})^{-1}\exp(-h_e^2/2\sigma_{zj}^2)\sum_m(q_{jm}/u_{jm}) \qquad (5)$$

Specific activity of tritium in the atmospheric moisture then can be calculated from χ_{AVG} divided by the absolute humidity of the atmosphere(H:g/m^3). $\quad C_a = \chi_{AVG}/H \qquad (6)$

Meanwhile, the removal of tritium from the atmosphere occurs primarily through the precipitation process. Assuming that tritium concentration in the rain is in equilibrium with that in the air, the effective equilibrium washout coefficient (s^{-1}) is described as

$$\Lambda = C_p\delta\left(\int_0^\infty \chi_{AVG}\,dz\right)^{-1} \qquad (7)$$

where C_p and δ represent the rain drop tritium concentration (Ci/cm^3) and the precipitation intensity(mm/s), respectively. The mean tritium concentration in the wind direction of sector i is then derived as

$$C_p = \Lambda\delta^{-1}\int_0^\infty \chi_{AVG}\,dz = NfQs(2\pi ux)^{-1} \qquad (8)$$

where s is the proportionality constant which is the ratio of washout coefficient and precipitation intensity(a/(mm s)). The expression for weighting and summing for particular wind speed class m is then

$$C_p = NfQs(2\pi x)^{-1}\sum_m(q_m/u_m) \qquad (9)$$

where q_m is the frequency of appearence of wind speed u_m.

The amount of deposition of tritium on unit area can be calculated for each precipitation intensity t as

$$B = NfA(2\pi x)^{-1}\sum_{mt}(q_{mt}\Lambda_t/u_m) \qquad (10)$$

where

B = burden per unit area(Ci/m^2)

A = activity emitted(Ci)

$\Lambda_t = \delta_t s$: washout coefficient for precipitation of intensity t.

It is noteworthy that in the vicinity of the source, where the value of σ_z is usually small, the ground level tritium concentration in the atmosphere is markedly depressed by the effect of the exponential term in the equations(4) and (5). In the equations(8) to (10), on the other hand, the exponential term no longer appears, and the tritium concentration in precipitation or the amount of tritium deposition per unit area is only inversely proportional to the source distance.

The results of the comparative calculation are shown in Fig.8 where tritium concentrations in air moisture(C_a) and in precipitation(C_p) are plotted as a function of the source distance for a release rate of 1 Ci/d. The input conditions are listed below.

stability category		: C
mean wind speed	u	: 2 m/s
source height	h_e	: 50,100,200 m
prop.const. for washout	s	: 1×10^{-8} a/(mm s)
number of sectors	N	: 16
absolute humidity of the atmosphere	H	: 12 g/m^3

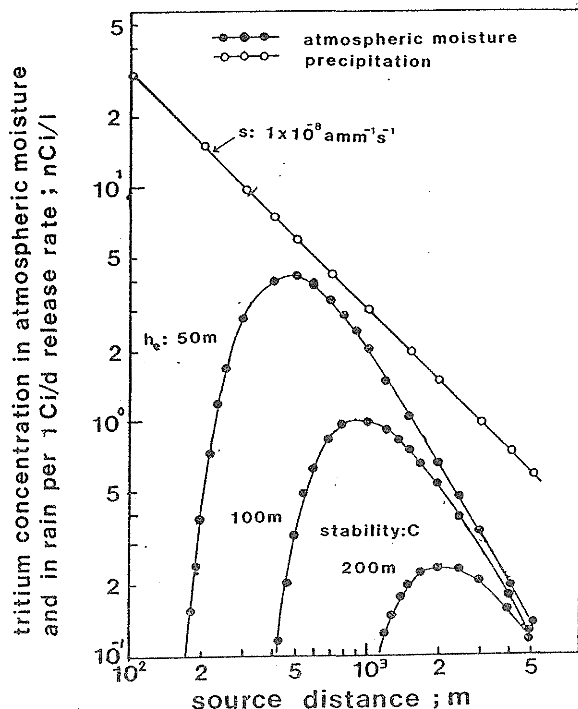


Fig.8: Source distance dependance of the tritium concentration in atmospheric moisture and precipitation as calculated according to the equations (4), (6) and (8).

Since the tritium concentration in precipitation is independent of the release height and stability category, whereas the maximum of the concentration in air moisture appears to decrease in magnitude at an ever increasing distance with growing source height.

For assessment of the radiological impact of the tritium released into the air,various methodologies have been proposed to calculate the dose to man. In some cases the doses due to ingestion of foods and drinking water at a particular location are assumed to be proportional to the concentration of tritium in air at that location. In others,the concentration of tritium in vegetation is assumed to be one-half of that in surrounding air. It is evident from the figure that these assumption will lead to an apparent underestimation of the dose in the vici-

nity of release point, particularly for high release height when contribution of washout deposition of tritium is neglected. The extent of underestimation as seen in the figure is found to be dependent on the release height. Further underestimation is depending on the source distance of the site boundary.
At a boundary of a source distance of 200 m, this underestimation amounts to more than one order of magnitude for a 50 m release height. At 1 km from the source, reasonable value is obtained for a 50 m release height but there is still underestimation of one order of magnitude for a 200 m release height.

It is concluded by the above consideration that the experimental determination of the washout deposition of tritium by precipitation is indispensable to assess the radiological impact of the tritium released into the atmosphere in the vicinity of the source .

NIRS-M-52, pp 316 - 332 , 1985

Environmental Tritium Transport Model. Application to the Dose Evaluation for Members of the Public

C.E.Murphy, Jr.

E.I.du Pont de Nemours & Co.
Savannah River Laboratory
Aiken, South Carolina 29808, U.S.A.

Abstact

A model of tritium dispersion and cycling in terrestial ecosystems surrounding an atmospheric source of tritium is presented. The model structure is described. Results of simulations for three release cases are used to illustrate the dynamics of the model. The three cases are; a release of 100% TH, a release of 100% THO, and a release of 50% TH and 50% THO. The TH release is characterized by having a lower total

---

DP-MS-84-117

This paper was prepared in connection with work done under Contract No. DE-AC09-76SR00001 with the U.S. Department of Energy. By acceptance of this paper, the publisher and/or recipient acknowledges the U.S. Government's right to retain a nonexclusive, royality-free license in and to any copyright covering this paper, along with the right to reproduce and to authorize others to reproduce all or part of the copy-righted paper.

retention of tritium in the ecosystem then the THO case. However, a greater fraction (but lower absolute amount) of the tritium in the TH release is found in the organic component of vegetation and the animals that feed on the vegetation.

Introduction

The study of tritium in the environment by the US-DoE has been an integral part of the study of the effect of radionuclides on the populations surrounding nuclear facilities. Since its inception in 1952, tritium has been studied at the Savannah River Plant (SRL) because of the facilities role in tritium production. Much of the early work was aimed at evaluating site specific tritium dose to ensure the safety of the working force and the general public. In the past ten years these studies were broadened under program funds supplied by the Office of Health and Environmental Programs (OHER) of DoE. The funding provided by OHER allowed us to do basic research on the cycling of tritium in the environment. Routine releases of tritium from the production facilities at SRL have resulted in trace amounts of tritium in the environment, providing an outdoor laboratory for the observation of tritium.

The tritium program at SRL has the objective of determining the dose from tritium releases to the surrounding population. This objective has been broadened under OHER research to developing the means to estimate the dose to the population surrounding any facility releasing tritium to the environment. In order to accomplish the objective of predicting tritium behavior in the environment under a wide variety of conditions, it is necessary to understand the processes which control

tritium transport. Therefore, a subsidiary objective to the above two objectives, is to understand the processes by which tritium is transported in the environment.

SRL research toward these objectives has included laboratory and field experiments aimed at understanding individual processes, study of patterns of tritium transport around manufacturing facilities, and synthesis of the information gained by developement of environmental systems models (1). The models have been useful in clarifying our ideas of how tritium acts in the environment, helping to set priorities for research, and developing a "hypothesis" which we can test against the distribution of tritium observed in the environment. The model presented here is the result of this research.

Model Structure

The model described here is based on an earlier model (2) of tritium dispersion. The model describes the steady state tritium activity at varying distances from a single source. In its present form it is a one dimensional model and does not resolve the distribution of tritium along an arc at a given distance from the source. The original model divided the environment into three compartments; the atmosphere, the vegetation water, and the soil. The version of the model presented in this paper also includes two grazers, dairy cattle and beef cattle. In addition, the biological compartments, i.e. vegetation and the cattle, are split into a water and an organic component. These additions were made to allow a food base to calculate dose to man with the model results. Detailed descriptions of the submodels used for each compartments are

presented below.

Atmospheric compartment

The source of tritium is from an atmospheric release at a given source elevation above the ground. The tritium is assumed to consist of a mixture of two forms, tritiated water (THO) and tritiated hydrogen (TH). Although the forms are defined as THO and TH, tritium is found in nature in mixtures including tritium, deuterium, and protium. Since these mixtures are dominated by protium, there is little loss in accuracy in treating the compounds as consisting of the normal abundances of tritium isotopes.

The tritium is advected from the source by the wind and is spread through the entire arc around the source. This approach results in an estimate of the average tritium concentration at a given distance from the source. The equation describing this process is:

$$C_a = Q / (2 \, pi \, u \, L \, r)$$

(1)

where C_a = the air concntration (Ci/m^3)

u = wind speed (m/d)

Q = the source strength (Ci/d)

L = the height of the mixed layer of the atmosphere (m)

r = the distance from the source (m)

pi = 3.1415 ...

This relationship is altered by correcting the ground concentration for the release height and vertical diffusion (3). The effects of exchange of tritium with the surface is modelled using the source depletion model, as suggested by Chamberlain (4).

The result of this model is an average value of concentration at a given distance from the source. This model was considered adequate for predicting long-term, averaage concentrations near SRP because the wind rose is near circular. If this is not the case the results should be compared to average measured concentrations where the averages are properly weighted by mixing depth and wind speed as suggested by equation (1).

Soil water compartment

The soil water exchange is modelled by a mass balance equation. The processes exchanging THO with the soil are rain, diffusion of THO vapor from the atmosphere, diffusion of TH to the soil where it is transformed to THO by microbial action, drainage of THO from the soil, and transpiration. The rainfall into the soil, transpiration into the vegetation, and drainage are processes where the THO moves by mass flow with the water in the system. Exchange of THO and TH with the soil are diffusion processes, which are characterized by a deposition velocity. The conversion of TH to THO in the soil is usually limited by diffusion in the soils found at SRP (5,6,7). Therefore the deposition velocity also determines the conversion rate. The mass balance for the soil is:

$$v_{ds} (C_{aw} - C_s H) + v_{dhs} C_{ah} + R r_o C_{aw} / H - T_r C_s - (R - T_r) C_s = 0 \quad (2)$$

where v_{ds} = deposition velocity of THO to the soil (d/m)

v_{dhs} = deposition velocity of TH to the soil (d/m)

C_{aw} = concentration of THO in air (Ci/m)

C_{ah} = concentration of TH in air (Ci/m)

C_s = concentration of THO in soil (Ci/m)

R = rainfall rate (kg/m^2 d)

r_o = washout ratio for THO

H = mixing ratio of water in air (kg/kg)

T_r = transpiration (kg/m^2 d)

The soil water submodel is coupled to the atmosphere through the air concentrations of TH and THO. Additional data required by the model are the rainfall and the transpiration rate. The deposition velocities are amoung the most difficult parameters to measure. Extensive measurements of the water balance of pine plantations, the dominant vegetation type at SRP, have provided sound estimates for use under SRP conditions. Where the necessary experiments have not been carried out it is usually possible to get adequate estimates from agricultural work done in the surrounding area.

Vegetation water compartment

The vegetation consists roots, stem, branches, and leaves. However, the key components of the vegetation are the leaves. The gaseous forms of tritium, THO vapor and TH, reach the inside of the leaves by diffusion from the atmosphere. The liquid THO is pulled up to the leaves from the soil by mass flow as water is evaporated from the leaves. Therefore, the leaf tritium mass balance is central to understanding tritium movement in the vegetation. The mass flow can be described by the equation(8,9,10):

$$m_{fs} [v_d (v_a - v_l)] = v_{dt} (v_{at} - v_{lt}) + v_{dh} C_t \qquad (3)$$

where m_{fs} = the mass fraction of tritium in the soil water

v_d = the exchange velocity for water vapor (day/m)

v_a = vapor density of water in the air (kg/m^3)

v_l = vapor density of water at the leaf surface, usually assumed to be the saturation vapor density at air temperature

v_{dt} = the exchange velocity of THO (day/m)

v_{at} = the vapor density of THO (Ci/m^3)

v_{lt} = the vapor density of THO at the leaf surface, assumed to be the saturation vapor density at leaf temperature multiplied by the mass fraction of tritiated water (Ci/m^3)

v_{dh} = the deposition velocity for TH (day/m)

C_t = the concentration of tritiated hydrogen in air (Ci/m^3)

This compartment is coupled to the atmospheric compartment by the air concentrations of TH and THO. The compartment is coupled to the soil water compartment through the mass fraction of tritium in the soil water. The most difficult parameter to determine in equation (3) is the leaf vapor density which requires a knowledge of leaf temperature. An estimate of leaf temperature and transpiration can be done by solving the leaf energy balance equation (10). In the model presented here estimates of the deposition velocity and the transpiration are used to estimate an average leaf temperature consistent with these values.

Uptake of TH by leaves appears to take place at a much slower rate than TH uptake by soil. However, the uptake of TH and its conversion to THO or tritiated organic matter does take place in, at least, some plants (11,12).

Vegetation organic compartment

From theoretical calculations it has been suggested that tritium could be accumulated or depleted in food chains or among the parts of organisms (13,14). However, measurements at the chemical, organism and ecosystem level do not show evidence of large departures from what would be expected from equilibrium dynamics (15, 16). Therefore, one would expect the tritium in the organic fraction to approach equilibrium with its environment. Garland (15) demonstrated a rejection factor of about 20% for wheat grown in a tritiated environment.

This picture is complicated by the fact that tritium may be in labile or nonlabile sites in the chemical structure of organic compound(17, 18). A simple example is in the synthesis of sugar by the photosynthetic system. When glucose is synthesized from carbon dioxide and water, the sugar produced is in equilibrium with the water with respect to tritium. Later these sugars may move to another environment where the THO concentration will change. In the new environment the hydroxyl groups in the sugar will exchange with the THO and equilibrium will be approached with the current THO environment. However, the carbon bounded tritium will not exchange and will remain at the relative abundance set at the time of synthesis. Therefore, the tritium concentration of organics has a "memory" but not a perfect "memory".

In an environment where more than one form of tritium exists, the situation is even more complicated. The specific activity of the different compounds may be very different and the activity of the organic matter may be a mixture of approaches to the different equilibrium status of each compound. We feel that we have detected such a situation at SRP. Tritium

in the organic fraction of leaves is higher than one would expect from the THO environment. However, the specific activity of TH in the environment is much higher than that of THO. A possible explanation of the higher organic tritium values is that there are synthetic processes in the plant that approach equilibrium with the TH(19). There is even reason to believe that atmospheric TH is responsible for higher than expected organic tritium at other locations(20).

In this model we have assumed that the increase in organic tritium activity is due to the presents of TH. In calculations the organic fraction is divided into labile and nonlabile fractions. The labile fraction is assumed to be at equilibrium with the vegetation water. The nonlabile fraction is assumed to have two components. One component is formed in equilibrium with THO in the leaf water and the other with the TH in solution in the leaf water. The partitioning of the two sources is at a ratio of 1:10000 for TH:THO. It is likely that any biochemical mechanism that actually produces this effect is not this simple, but in lieu of a better scheme, this approach provides realistic estimates of the tritium content of the organic fraction under SRP conditions.

Grazer compartments

There are two grazer compartments. The dairy cow compartment is included because of the importance of milk in the diet of infants. The beef cattle compartment is one example of a source of meat for adults. The basic assumptions in the modelling of these compartments are; the concentration of tritium in the cattle body water is determined by the water

balance of the animals, and the concentration of tritium in the organic component of the cattle is determined by both the concentration in the food and the concentration in the body water.

The water balance equation used to calculate the body water content is:

$$C_{cw} = (w_v\ C_v + w_o\ C_{vo} + w_d\ C_{dw} + 2.\ B\ C_a/H)/(B/H + w_e) \qquad (4)$$

where

w_v = volume of THO consumed in vegetation (Ci/day)

w_o = volume of THO from oxidation of food (Ci/day)

w_d = volume of THO from drinking water (Ci/day)

w_e = volume of THO eliminated from body (Ci/day)

B = breathing rate of cattle (m^3-air/day)

C_v = concentration of THO in vegetation water (Ci/day)

C_{vo} = concentration of tritium oxidized from food in THO units (Ci/m^3-water)

C_{dw} = concentration of THO in drinking water (Ci/m^3).

Anspaugh et al. (21) have compiled the rates of water input and elimination necessary to make calculations of trtium exchange for dairy and beef cattle. The dominant input paths are the water ingested in food and drinking. The factor of two before the breathing rate is to account for absorption of THO through the skin. However, even with this doubling of the rate of exchange with the atmosphere, the path from atmosphere to body water is the least important, quantitatively, of all the paths used in the model.

The concentration of the organic fraction is modelled by the equation:

$$C_{co} = f_f\ C_{vo} + (1. - f_f)\ C_{dw} \qquad (5)$$

where

f_f = the fraction of nonlabile tritium in the food. Experiments with cattle fed THO indicate that the milk solids produced have a tritium concentration about 0.5 of the THO. When cattle were fed tritiated hay the milk solids had a concentration that was about 0.5 of the concentration in the hay (22). Both of these experiments suggest that about one half of the hydrogen sites in the food are nonlabile. Experiments with young calves fed tritiated milk suggest a factor of 0.4-0.5 nonlabile/labile hydrogen sites. In the simulations shown later in this paper a value of 0.5 was used for milk from dairy cattle and 0.4 for meat from beef cattle.

Results

The modelling equations were coded in the simulation language CSMP (23). Three cases are presented here to illustrate the characteristics of the model. The results have been compared to environmental survey data taken at SRP in an earlier publication (2). The three cases are atmospheric releases of 1) 100% THO, 2) 100% TH, and 50% TH - 50% THO. The results are presented in table 1. The values of tritium concentration (uCi/m^3-water) are scaled against the size of the release (kCi/day).

The THO release is characterized by having the highest concentration in the air moisture. The soil moisture is less because of the imposed washout ratio of 0.5 taken from environmental survey data (24). The vegetation water concentration is between the concentration of the air and the soil moisture. The vegetation organic fraction is lower than the water fraction because of the 0.8 rejection factor for the

Table 1. The results of model simulations for three cases of atmospheric tritium releases are presented; 100% TH, 100% THO, and 50% each TH-THO. The results show the specific activity ratio of the model compartments in (uCi/kg-water)/(Ci released/day) at distances from 10 to 50 kilometers from the source.

	Distance					
Compartment/	10 km			50 km		
Case	THO	TH	TH-THO	THO	TH	TH-THO
Atmosphere	6.9	.07	3.5	1.3	.05	.68
Rain	3.5	.04	1.8	.75	.02	.34
Soil	4.4	3.9	4.1	.83	.75	.79
Vegetation\						
Water	6.6	3.5	5.1	1.3	.69	.97
Organic	5.3	22.	13.	1.0	4.1	2.6
Dairy Cattle						
Milk	6.9	.15	3.5	1.3	.06	.68
Milk Solids	6.1	11.	8.5	1.2	2.1	1.6
Beef Cattle						
Water	6.9	.13	3.5	1.3	.06	.68
Meat	6.2	9.4	8.0	1.2	1.8	1.5

synthesis process. The model also predicts that the animal compartments will be similar to the vegetation. The organic fractions are similar to the water fractions because the environmental water and the food are very similar in concentration levels. One assumption made in these calculations is that the surface water used for drinking has the same concentration as the rain water. If well water was used for drinking, the concentrations could be substantually reduced because the well water could be old enough to allow significant decay of tritium before use, even in cases where a steady state had been established with the well water.

The TH release is quite different in character than the THO release. The Highest concentration of THO is found in the

vegetation organic component. The vegetation organic component has a higher specific activity than the soil water even though the uptake by the soil is greater. This is because of the greater dilution of the soil THO by rain water. The vegetation water is somewhat lower than the soil moisture because of differential loss of THO to the atmosphere. The organically bound tritium in the vegetation is the most important input to the animal tritium compartments. The model assumes no direct conversion of TH to THO in the animals. The conversion in the soil does provide a source for some THO in the atmosphere. However, most of the tritium in the body water comes from the organic component of the food. The direct incorporation of this organic material results in a higher organic component than water component in both milk solids and beef meat.

The combined release of equal quantities of TH and THO show no surprises. The characteristics of the release are a combination of the other releases. Thesoil moisture is higher than the air moisture because of the conversion of TH to THO. The vegetation water is between the concentration of the air and the soil moisture. The vegetation organic material is elevated by the direct incorporation of a small amount of TH. The animal compartments reflect the higher organic tritium in their food by an elevated level of their organic components.

Conclusions

The results of the model point out some things that should be considered in making dose estimates for atmospheric tritium releases. The chemical species containing the tritium will have a large influence on the characteristics of the tritium found in the environment. The total amount of tritium retained in the

system will be less for a release of TH than for an equal activity release of THO. Even though the concentration of tritium in the organic components of food, in the TH release case, is greater than the level in the THO case, the smaller amount of organic tritium ingestion and the much lower THO activities would tend to keep the dose to man substantually below the dose from THO releases.

However, the fact that the tritium in the organic component can be higher than that in the body water of human food under TH release conditions suggests that an estimate of dose, based on urine samples or other means of determining body water tritium concentration, would under estimate the dose to man. Under these conditions it would be prudent to also determine the organic tritium concentration of affected populations.

REFERENCES

1. C. E. Murphy Jr., C. W. Sweet and R. D. Fallon, Tritium transport around nuclear facilities. Nuclear Safety 23, 677-685 (1982).

2. C. E. Murphy Jr. and M. M. Pendergast, Environmental transport and cycling of tritium in the vicinity of atmospheric releases. In Behavior of Tritium in the Environment, pp 361-372, IAEA,Vienna,1979.

3. J. H. Novak and D. B. Turner Jr., An efficient Gaussian-plume multiple-source air quality algorithm. J. Air Pollution Control Assoc. 26, 570-575 (1976).

4. A. C. Chamberlain, Aspects of Travel and Deposition of Aerosol and Vapor Clouds. British Report AERE-HP/R-1261.

5. C. W. Sweet and C. E. Murphy Jr., Oxidation of molecular tritium by inttact soils. Environmental Science & Technology 15, 1485-1487 (1981).

6. R. D. Fallon, Molecular tritium uptake in southeastern U. S. soils. Soil Biol Biochem. 14, 553-556 (1982).

7. J. A. Garland and L. C. Cox, The absorption of tritium gas by English soils, and plants and the sea. Water, Air and Soil Pollution 14, 103-114 (1980).

8. F. Raney and Y. Vaadia, Movement and distribution of THO in tissue water and vapor transpired by shoots of Helianthus and Nicotiana, Plant Phys. 40, 383-388 (1965).

9. Y. Belot, D. Ganthier, H. Camus,and C. Caput, Prediction of the flux of tritiated water from the air to plant leaves, Health Phys. 37, 575-583 (1979).

10. C. E. Murphy Jr., The relationship between tritiated water activities in air, vegetation, and soil under steady-state conditions. accepted for publication in Health Physics.

11. C. E. Murphy Jr., A. L. Boni, and S. P. Tucker. The Conversion of Gaseous Molecular Tritium to Tritiated Water in Biological Systems. DP-1422, E. I. du Pont de Nemours & Co., Inc., Savannah River Laboratory, Aiken, S. C., 1976.

12. J. C. McFarlane, Tritium accumulation in lettuce fumigated with elemental tritium. Envir. and Exp. Botany 18, 131-138 (1978).

13. D. G. Jacobs D. G., 1968, Sources of Tritium and Its Behavior upon Release to the Environment. USAEC, Oak Ridge, Tennessee, 1968.

14. R. E. Wesson Jr., Kinetics of tritium in biological systems. In Tritium (A. A. Moghissi and M. W. Carter Eds.) pp 289-303. Messenger Graphics, Pbl., Phoenix, Arizona, USA, 1973.

15. H. D. Bruner, Distribution of tritium between the hydrosphere and invertebrates. In Tritium (A. A. Moghissi and M. W. Carter Eds.) pp 303-314. Messenger Graphics, Pbl., Phoenix, Arizona, USA, 1973.

16. J. A. Garland, Incorporation of tritium in grain plants. Health Physics 36, 35-38 (1979).

17. V. J. Frilette, J. Hanle and H. Mark, Rate of exchange of cellulose with heavy water. Amer. Chem. Soc. 70, 1107-1113 (1948).

18. Y. Belot, J. Guenot, C. Caput and F. Bourdeau, Incoporation of tritium into organic matter of terrestrial plants exposed to tritiated-water releases of short duration. Health Physics 44, 666-668 (1983).

19. C. W. Sweet and C. E. Murphy Jr., Tritium deposition in pines and soil from atmospheric releases of molecular tritium, Envirn. Sci. Tech. 18,358-361 (1984).

20. D. C. Bogen and G. A. Welford, Fallout tritium distribution in the environment. Health Physics 30, 203-208 (1976).

21. L. R. Anspaugh, J. J. Koranda, W. L. Robison, and J. R. Martin, The dose to man via food-chain transfer resulting from exposure to tritiated water vapor. In Tritium (A. A. Moghissi and M. W. Carter Eds.) pp 405-422. Messenger Graphics, Pbl., Phoenix, Arizona, USA, 1973.

22. J. van den Hoek, R. Kirchmann, and N. B. Juan, Transfer and incorporation of tritium in mammals. In Behavior of Tritium in the Environment, pp 433-444, IAEA,Vienna,1979.

23. IBM, Continuous System Modeling Program III (CSMP III) Program Reference. IBM World Trade Corporation, New York, 1975.

24. C. Ashley, C. C. Zeigler, and D. R. Quisenberry, Tritium in the Environment at the Savannah River Plant. DPSPU 79-30-9, E. I. du Pont de Nemours and Company, Aiken, SC, 1979.

NIRS-M-52, pp 333 - 345, 1985

Experience of Tritium Monitoring for Work Place and Environment in JAERI

M.Murata, K.Obata, T.Ohhata, H.Katagiri,
H.Yamamoto, M.Okubo and Y.Yoshida

Department of Health Physics
Japan Atomic Energy Research Institute
Takai-mura, Naka-gun, Ibaraki-ken, 319-11, Japan

Abstract

At Tokai Research Establishment, there are two heavy water moderated research reactors JRR-2 and JRR-3 (tritium inventory 4×10^4 Ci). Presently, for the fusion research program, a test production of tritium at 100 Ci/Y is carried out at Radioisotope Production Facility. At Fusion Neutronics Source Facility, 10^3 Ci of tritium targets are always used for generation of 14 MeV neutrons. Tritium Process Laboratory is under construction, where 10^5 Ci of tritium are expected to be handled for tritium systems studies.

For work place and stack air monitoring, ionization chamber type monitors are used for continuous measurement of total tritium concentration. An adsorbent or a cold trap is used for precise measurement of HTO or DTO, and catalytic oxidation + adsorbent for HT or DT.

An environmental tritium monitoring was started in 1980 for river

water, sea water and drinking water. Since 1984, in addition to the above items, tritium concentrations in air (HT/HTO discrimination), rain water, and water of pine needles are measured routinely, because of an increase of amount of handling tritium. Correlation is being studied between the tritium concentrations in the environmental samples and the air concentrations calculated from the data of tritium release and the meteorological conditions.

The present paper describes the present status of the work place and the environmental monitoring techniques of tritium including some experiences in Department of Health Physics at Tokai Research Establishment in JAERI.

1. Tritium handling facilities at Tokai Research Establishment

Among many facilities at Tokai Research Establishment, the following facilities are concerned with tritium (Table 1).

At Tokai Research Establishment, the number of radiation workers is about 1,800 (in 1983), of which about 300 (in 1983) are workers handling tritium or working under a tritium atmosphere.

Table 1 Tritium handling facilities in Tokai Research Establishment

Tritium handling Facility	Sources	Tritium Inventory(Ci)
JRR-2 JRR-3	Heavy water moderator	9000 28000
Radioisotope Production Facility	Production of tritium	1400
Fusion Neutronics Source Facility	Neutron source target	9600
Tritium Process Laboratory	(Under construction)	(10^5)
Van de Graaff	Target	
Waste Treatment Facility	Waste	
Research Laboratories	Laboratory experiment	10

* July 1984

(1) Reactors

At Tokai Research Establishment, there are two heavy water reactors,

that is, JRR-2 and JRR-3. JRR-2 is an enriched uranium heavy water reactor. About 9 tons of heavy water are used as the moderator. The concentration of tritium in the heavy water increases every year. Presently, the concentration is about 1 mCi/ml. JRR-3 is also an enriched uranium heavy water reactor. About 28 tons of heavy water are used as the moderator. The tritium concentration is similar to that of JRR-2. Tritium inventories of JRR-2 and JRR-3 are about 9,000 Ci and 28,000 Ci, respectively.

(2) Production of tritium for fusion research

Recently, for the fusion research and development program, a test production of tritium on 100 Ci level is carried out. The source material is Li-Al alloy, and the final product is uranium-absorbed tritium. During the purification process, tritium is handled as a gas. At the present time, annual production of tritium is several hundred curies.

(3) Van de Graaf accelerator

Tritium was frequently used as a target. The target tritium was either in the gaseous state or absorbed on a metal. In most cases the amounts of tirtium handled were on the curie level. Leakage of the tritium sometimes occured in the target room.

(4) Fusion Neutronics Source Facility

14 MeV of neutrons are generated by bombarding deutrons to a tritium target. 14 MeV neutrons are needed for experiments concerning fusion neutronics. About 10^3 curies of tritium target is always used.

(5) Tritium Process Laboratory

Tritium Process Laboratory is under construction. In this laboratory, 10^5 curies of tritium is expected to be handled for tritium systems studies.

(6) Other facilities

Contamination with tritium is also observed occasionally in the waste

treatment plant and research laboratories.

2. Regulations for protection of the workers and public from tritium

Table 2 shows basic standards for the workers and the public specified by the Japanese law. The values shown in the table are the same as those given in the ICRP Publication 2, except that only one value of (MPC)a specified for tritiated water vapour (HTO, etc.) by the publication is given, irrespective of chemical form, HTO or HT, etc.

Basically, (MPC)a (or Derived Air Concentration according to the ICRP Publication 30) should be given depending on chemical forms in the ICRP. The design goal dose for the public based on the ALARA concept is actually required to be less than 5 mrem/y from both gaseous and liquid effluents, although this is not officially specified by the laws. And release rate control level (Derived Working Limit DWL) is decided so as to meet the design objective for the operation.

Table 2 Basic standards specified by the laws for the public and workers

	Uncontrolled area	Controlled area
1) Dose limit	500 mrem/y	5000 mrem/y
2) (MPC)a	2×10^{-7} μCi/cm^3	5×10^{-6} μCi/cm^3
(MPC)w	3×10^{-3} μCi/cm^3	7.5×10^{-2} μCi/cm^3
3) Regulation for effluents		
a) Concentration, daily or quarterly average		
i) $< \frac{1}{10}$ (MPC)a,w at a release point		
ii) $< \frac{1}{10}$ (MPC)a,w at a site boundary		
b) Dose 500 mrem/y		

3. Work place and environmental monitoring

The Tritium monitoring techniques in JAERI were developed mainly based on the experiences of those in the heavy water moderated research reactors, JRR-2 and JRR-3 and handling of the tritium gas targets of accelerators.

(1) Air monitoring techniques

In Table 3, monitoring techniques of airborne tritium in JAERI are summarized with the detection limits.

Ionization chamber type monitors are used for continuous measurement of total tritium concentration in air, independent of the chemical form. For the stack monitors in the recent facilities, ionization chambers with a large volume and gamma compensation are used to obtain the detection range from 10^{-7} to 10^{-2} $\mu Ci/cm^3$. In addition to continuous measurements by ionization chamber type monitors, a method by condensation, adsorption or bubbling is used for precise measurements of tritiated water vapor in air such as HTO or DTO. In these three methods, in general, periodical measurements are made through continuous collection of water vapor samples by condensation, adsorption or bubbling followed by the liquid scintillation counting.

In the cold trap method, as well known, the concentration of tritiated water vapor in air is determined from the measured radioactivity of tritium in the condensed water sample, the relative humidity and the density of saturated water vapor which is calculated depending on the temperature of sampling air. And this method is applied mainly for measuring the low concentration of tritiated water vapor in working area. The adsorption method is used mainly for obtaining the average concentration of tritiated water in air for a long period such as in stack monitoring.

Water collected in adsorbent, silica-gel, is extracted by heating or dipping it in water. Procedure of the dipping is simpler than the heating and so is used in routine monitoring, though the minimum detectable amount at the dipping method is approximately one order of magnitude greater than at the heating method.

For the measurement of very low level of tritium in the form of hydrogen gas, the technique of catalytic oxidation followed by adsorption

is also used. The detection limits shown in the table are for that liquid scintillation counting of $10^{-6} \sim 10^{-7}$ μCi/ml.

Table 3 Monitoring Techniques of Airborne Tritium in JAERI

Sampling method	Instrument or Sampling material	Detection limit ($\mu Ci/cm^3$)
Direct sampling	Ionization chamber (Volume: 1500 cm^3)	2×10^{-6}
	Ionization chamber type monitor	$10^{-5} - 10^{-7}$
Condensation	Cold trap- L.S.C.*	10^{-11}
Adsorption	Silica gel- L.S.C.*	$10^{-11} - 10^{-12}$
Bubbling	Water bubbler- L.S.C.*	10^{-7}
Oxidation + adsorption	Catalyzer + adsorber - L.S.C.*	$10^{-10} - 10^{-12}$

* Liquid scintillation counter

(2) Surface contamination monitoring technique

Monitoring of tritium surface contamination has been made through counting smear samples with a 2π gas flow counter or a liquid scintillation counter. Filter papers loaded with 25 wt% glycerol in ethanol are used for the smear in order to hold the tritium collected in the samples and also to count fairly efficiently.

(3) Liquid effluent monitoring techniques

For liquid effluent monitoring, the liquid samples are taken for measurement before release from the liquid waste tanks in the facilities to sea. Measurement is made by liquid scintillation counting of the water sample usually without treatment such as distillation.

(4) Monitoring system of gaseous effluents

Some examples of tritium monitoring system for gaseous effluents are shown. Fig. 1 shows the tritium monitoring system in JRR-2, (the heavy water moderated research reactor) whose tritium concentration in moderator is about 1 mCi/ml at present. The gas monitor shown in the figure measures mainly ^{41}Ar, main source of this reactor, and release of airborne tritium is obtained through measurement of tritiated water sample collected continuously with a silica-gel column, each month. Air contamination with tritium in the working area is monitored by the installed ionization chambers and also measured periodically by the condensation method with a cold trap, although it is not shown in the figure.

Fig. 2 shows the tritium monitoring system in the Fusion Neutronics Source Facility (FNS), where 1000 Ci of tritium is handled as a metal target. The stack tritium monitor is made of ionization chamber of 30 litres with gamma compensation, whose minimum detection limit is about 3×10^{-7} μCi/cm^3. Fig. 3 shows the tritium monitoring system for Tritium Processing Laboratory (TPL) under construction. In FNS and TPL, tritium in the chemical form of HTO is continuously sampled using the silica-gel adsorber and periodically measured every two weeks. The sampling system of catalytic oxidation and adsorption is used as back-up for a tritium monitor.

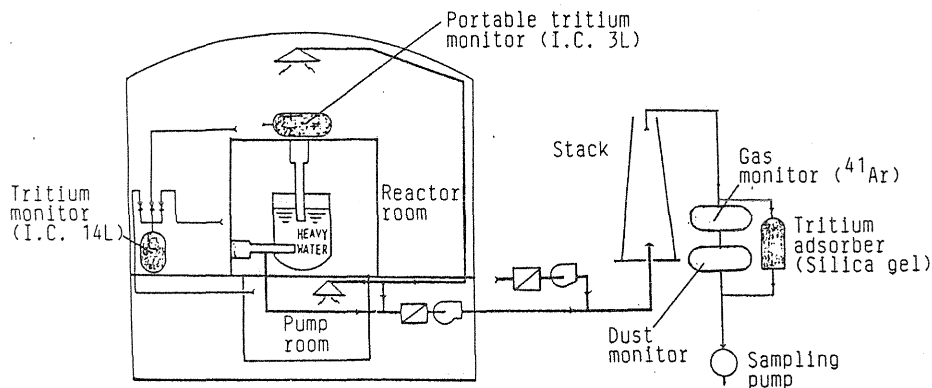


Fig.1 Tritium monitoring system in JRR-2

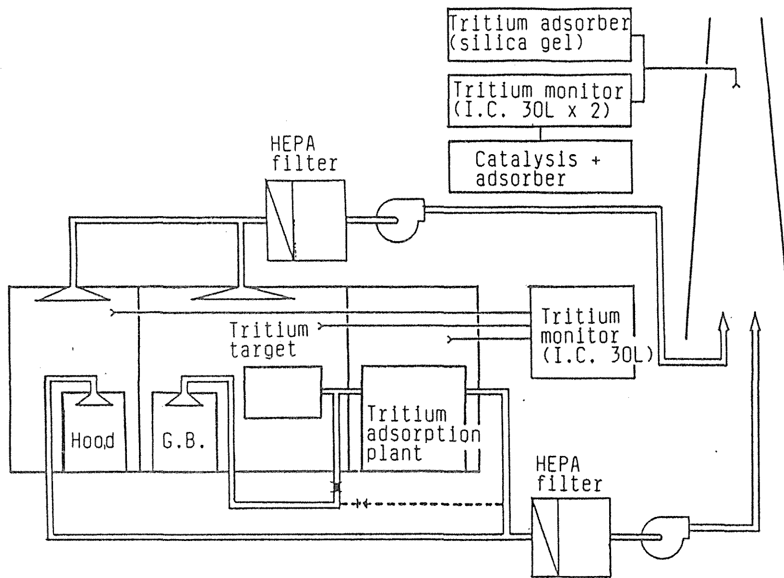


Fig.2 Tritium monitoring system in Fusion Neutronics Source Facility

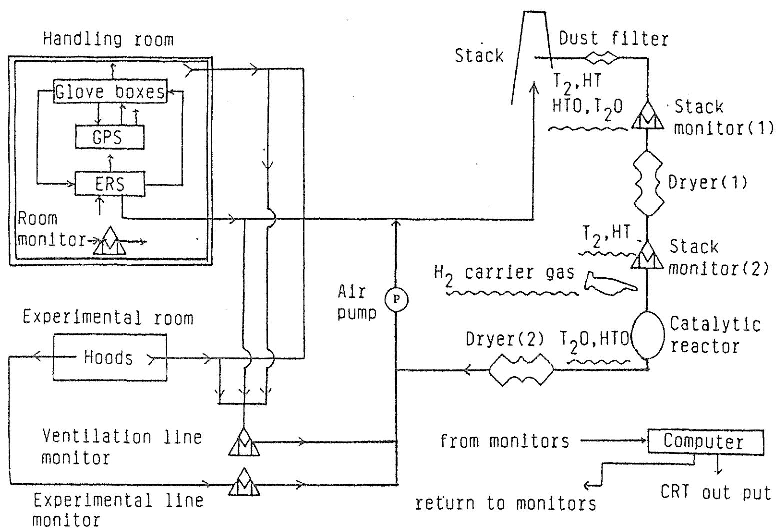


Fig.3 Radiation monitoring system of the Tritium Process Laboratory

(5) Environmental monitoring

An environmental tritium monitoring was started in 1980 for river water, sea water and drinking water. Since 1984, in addition to the above items, tritium concentrations in air (HT/HTO discrimination), rain water, and water of pine needles are measured routinely, because of an increase of amount of handling tritium.

The sampling locations of the environmental samples are shown in Fig. 4 along with the locations of the monitoring posts and the stacks of JRR-2, JRR-3, FNS and TPL.

The tritium sampling points are located down-wind in the most frequent wind direction.

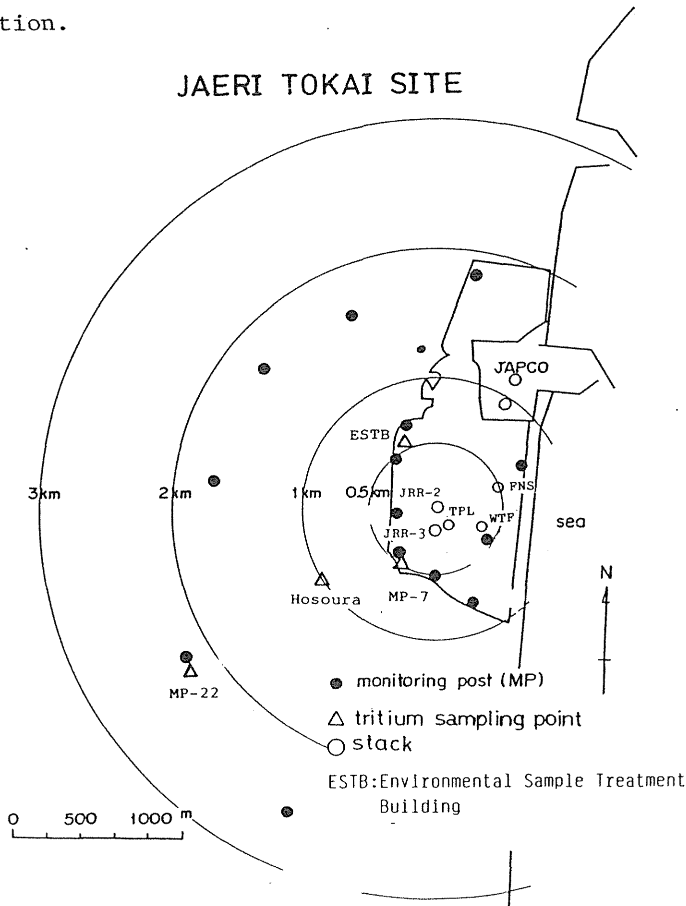


Fig.4 Monitoring points of the environmental radiation and environmental tritium samples

4. Monitoring experience

(1) Monitoring data

Tritium atmospheric release and concentration in workplaces of research reactors, Radioisotope Production Facility (RPF) and FNS facility are shown in Table 4. Atmospheric releases from JRR-2 and JRR-3 are 11-44 Ci/y and 7-30 Ci/y respectively in recent three years, and those from RPF and FNS are, respectively, 2 Ci/batch and 0.4 Ci/8 months in the form of

tritium gas. Liquid release of tritium from these facilities is several curies per year. Tritium concentration in work places in JRR-2 and JRR-3 reaches up to the order of 10^{-4} μCi/cm^3. In RPF and FNS, tritium air concentrations in work places have been lower because of the use of local exhaust system. Individual doses due to tritium inhalation are usually insignificant in comparison with external radiation doses in JAERI. A maximum dose of the persons inhaling tritium during gas targets handling was 380 mrem.

Table 4 Tritium release from facilities in JAERI

Facility	Amount of tririum	Atomospheric release	Concentration in workplace	Remarks
JRR-2	1 mCi/cm^3(D_2O)	11 - 44 Ci/y	~ 3 x 10^{-4}μCi/cm^3 *1	*1 Repair work of primary coolant system
JRR-3	1 mCi/cm^3(D_2O)	7 - 30 Ci/y	~ 3 x 10^{-4}μCi/cm^3 *1	
Radioisotope Production Facility	80 Ci(irradiated Al-Li)	- 2 Ci/batch	< 2 x 10^{-6}μCi/cm^3 *2	*2 Operation area
Fusion Neutronics Source facility	1000 Ci (metal target)	0.4 Ci/8 months	< 1 x 10^{-6}μCi/cm^3 *3	*3 Exchanging target in glove box

(2) Relation between contamination of tritium in air and on surface at heavy water moderated reactor[1]

The relationship between the tritium concentration in air and the tritium contamination on surface was studied in the reactor room of JRR-2.

A floor (epoxy resin) and sample plates (acrylic resin and aluminum) were exposed to the tritium-contaminated-air in the reactor room of JRR-2. Smear samples were taken from the surfaces and analyzed.

It is seen from the Fig. 5 that approximately linear reiationship was shown, irrespective of the surface materials. The ratio of the surface contamination (μCi/cm^2) to the concentration in air (μCi/cm^3) was 0.81 cm.

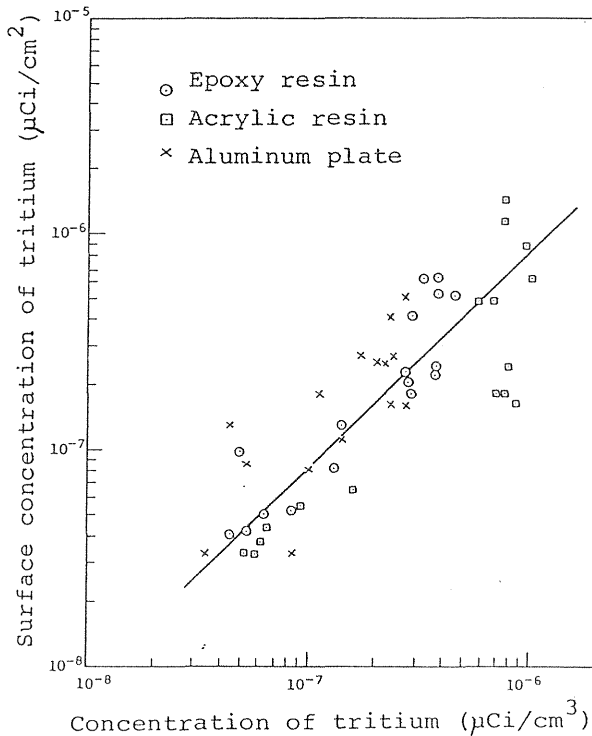


Fig.5 Relation between contamination of tritium in air and on surface

(3) Distribution of tritium through the thickness of concrete in JRR-3[2]

Powdery concrete samples were taken from the wall and floor of the reactor building by drilling.

Concrete powder was immersed for a week in water to leach out tritium. The wall and floor had been exposed to tritiated heavy water and its vapor for 20 years.

Fig. 6 shows a typical distribution of tritium through the thickness. It is seen that the tritium concentration in concrete decreases exponentially with increasing thickness.

5. Personal protective equipments

For the protection of workers from the air contamination with tritiated water, ordinary protective equipments developed for aerosol and iodine protection have been used.

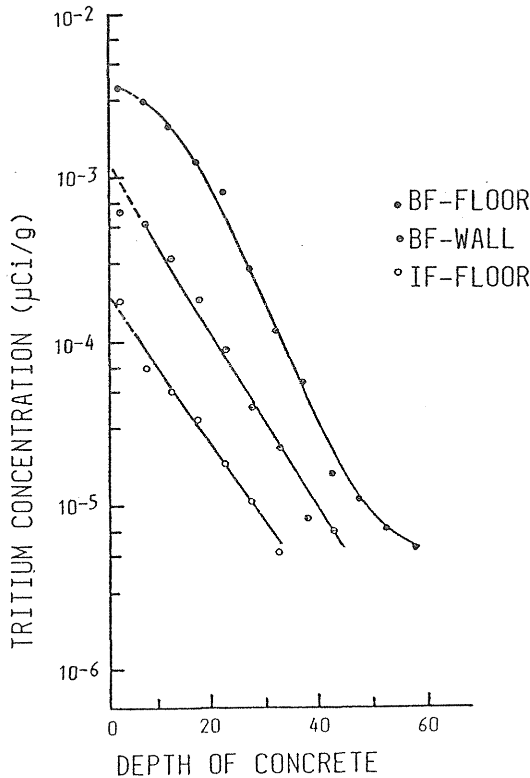


Fig.6 Depth distribution of tritium concentration in concrete

A charcoal cartridge is used for adsorption of HTO vapor in a full-facepiece mask at the concentration level of $(MPC)_a$ or less. Above $(MPC)_a$ to 100 $(MPC)_a$ level, a supplied air full-facepiece mask is often used with a vinyl anarak in repairing work of the primary coolant pipes of the heavy water reactors. At still higher contamination levels, an air supplied whole-body suit is worn. Fig. 7 and Fig. 8 show the suit and the air-supply line in the hot laboratory, respectively.

Because, no special designed protective equipment is available for tritium in JAERI, experimental studies are now planned to measure the permeation rate of tritiated water for the modern polymeric films and to measure the total protection factor of the air supplied whole-body suit for tritium.

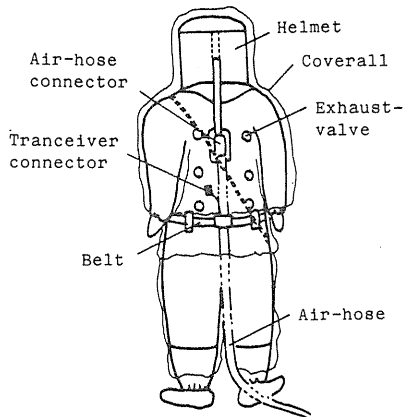


Fig.7 Supplied air whole-body suit
Air : - 500 l/min
Press. : 10-20 mmAq
Temp. : 18 °C
R.H. : 43 %

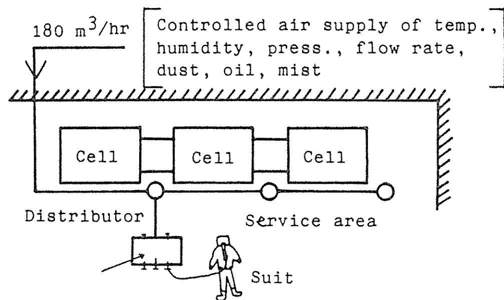


Fig.8 Air supply system for whole-body suit

REFERENCES

[1] T. Hayasaka, Health Physics in JAERI, No. 22, JAERI-M 83-134, 107 (1980)

[2] Y. Izumi, Y. Takaba, and T. Saito, Health physics in JAERI, No. 25, JAERI-M 83-134, 143 (1983)

NIRS-M-52, pp 346 - 356, 1985

Experience of Tritium Protection and Exposure of Workers in JAERI

J.Akaishi, H.Fukuda, T.Hattori and S.Suga

Bioassay Division, Japan Atomic Energy Research Institute
Tokai-mura, Ibaraki-ken, 319-11, Japan

ABSTRACT

Individual monitoring for internal contamination of tritium was started in 1963. The monitoring program consists of the following : (1) routine monitoring every three months for selected personnel, and (2) special monitoring for assessing internal contamination and estimating the committed dose equivalent.

In the JAERI, there are many facilities where tritium is handled;including heavy water moderated reactors,JRR-2 and JRR-3, and Van de Graaff accelerator. About 90 % of the personnel subjected to the special monitoring have worked in these three facilities. A total of about 2000 persons have received the routine monitoring up to the end of fiscal 1983. Seven persons were subjected to the bioassay and special

monitoring. This indicates the adequacy of radiation control for the workplaces. A cumulative total of about 370 persons had received the special monitoring. Doses were below the detectable level for 28 %, less than 10 mrem for 51 %, and between 10 and 250 mrem for 21 %; only one person received a committed dose equivalent of 380 mrem. The variation in biological half life of incorporated tritium was studied for the 41 traceable cases which received special monitoring. The half-lives were in the range of 5 to 17 days, with 9.4 ± 2.7 days as the average. There was good agreement between estimated doses observed by exhaled air measurement at workplace and those observed by urine analysis.

INTRODUCTION

This paper presents about 20 years' experience of individual monitoring for internal contamination of tritium at the Japan Atomic Energy Research Institute.

The Japan Atomic Energy Research Institute was established in 1956, and research activities in the Tokai Institute were started in 1958.

Among many facilities in Tokai Institute, the following 5 facilities are the main ones concerned with tritium exposures; 1, tritium production laboratory, 2, and 3, heavy water reactors Japan Research Reactor-2 and -3, 4, Van de Graaff accelerator, and 5, waste treatment plant.

In Tokai Institute, the number of radiation workers is about 1800 in 1983, of which about 300 workers are handling tritium or working in air contaminated with tritium.

PRODUCTION AND HANDLING OF TRITIUM AT TOKAI INSTITUTE

Tests of tritium production, for fusion research, are generating 100 Ci of tritium per test. During the purification process, tritium is handled as a gas. At the present, the annual production of tritium is several hundred curies.

In Tokai Institute, there are two heavy water reactors, JRR-2 and JRR-3. In JRR-2, about 9 tons of heavy water is used as a moderator.

The concentration of tritium in the heavy water increased each year as shown in Fig. 1 . At the present, the concentration is about 1 mCi/ml. During nearly 20 years of operation, small leakage of the heavy water has occurred occasionally.

In JRR-3, about 15 tons of heavy water is used as a moderator. The tritium concentration is similar to that of JRR-2.

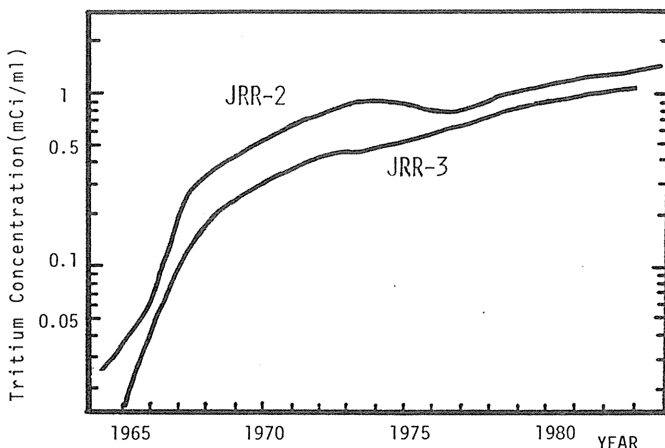


Fig.1 Tritium concentration of heavy water reactors, JRR-2 and JRR-3.

Tritium is frequently used as a target in the Van de Graaff accelerator. The target tritium is either gaseous or metal adsorbed form. In most cases, the amount of tritium handled is at about the curie level. Small leakage of the

tritium sometimes occurred in the target room.

Besides these facilities, contamination is also observed occasionally in the waste treatment plant.

In JAERI, tritium is handled in the form of tritiated water or tritium gas. Our experience of internal contamination has been only for tritiated water.

The monitorings of tritium in air at the work place has already been presented in the preceeding paper.

PROTECTION OF THE WORKERS FROM TRITIUM CONTAMINATION

An ordinary cartridge type mask does not provide adequate protection for both tritium gas and tritiated water vapor. Therefore, an air supplied, full face mask is usually used for low level air contamination (10(MPC)a - 100((MPC)a), and air supplied suit with a full-face mask is used for high level air contamination (over 100(MPC)a).

When internal contamination is suspected, tritium concentration of the worker's exhaled air is measured at the work place, as the preliminary individual monitoring. When the internal dose of more than 10 mrem is estimated, by the exhaled air measurement, internal monitoring by bioassay is carried out.

INDIVIDUAL MONITORING OF INTERNAL CONTAMINATION

Both the routine and special monitoring are carried out by bioassay.

(1) Routine Monitoring

The purpose of routine monitoring is to check the significant incorporation of radioactivity into the body. At present, for tritium, 10 mrem is adopted as the significant

exposure level for internal contamination at JAERI. The number of workers who have a possibility of tritium contamination is about 300. However, except for the group participating in the test production of tritium, the level of tritium handled is very low. In addition, the monitoring of workplaces and protection are carried out very strictly. For these reasons, individual monitoring is not routinely carried out for the all of workers who have a possibility of tritium contamination. At present, about 70 workers, more than one worker from each group, are picked as subjects for routine monitoring. This routine monitoring is carried out every three months.

(2) Special Monitoring

The purpose of special monitoring is to estimate the body burden, and a committed dose where necessary. This special monitoring is carried out for the following workers: a, workers found by routine monitoring to have a significant contamination, b, workers who had an accidental intake of tritium, c, workers who are suspected of intake of tritium.

For the measurement of tritium concentration in the body water, urine is generally sampled. Water collected from exhaled air or saliva is sometimes used as the sample. For urine analysis, usually 0.2 ml is taken as a sample, and mixed with 15 ml of scintillator. After several hours, the tritium activity is measured by a low-background liquid scintillation counter for 10 or 30 minutes. In 10 minutes counting, the detection limit is about 10 pCi for urine or other water samples.

ESTIMATION OF INTERNAL DOSE

The dose estimation is based on the method recommended by ICRP.

The committed dose equivalent is calculated by the formula

$$D = 51.2 \cdot (\varepsilon / m) \cdot q_0 \cdot \int_0^{50y} R(t)dt \text{ ------------------ (1)}$$

where, D is committed dose equivalent (rem/50 years), ε, effective energy of tritium (0.006 MeV), m, mass of the critical organ (body water, 43 kg), q_0, amount of initial incorporation (μCi), R(t), a retention function ($e^{-0.693t/T}$), and T, effective half-life (day).

Based on the assumption that the tritiated water is uniformly distributed in the body water, the amount of initial incorporation, q_0, is calculated by equation

$$q_0 = U_0 \cdot m \text{ ------------------------------------ (2)}$$

where, U_0 is initial concentration of tritium in the body water in μCi/g.

Combining equation (1) and (2), equation (3) is obtained.

$$D = 51.2 \cdot (\varepsilon/m) \cdot U_0 \cdot m \cdot (T/0.693) = 0.44\, U_0 \cdot T \text{ ---- (3)}$$

In this equation, effective half-life T is usually determined by follow-up measurement. If the follow-up measurement is not possible, the value recommended by ICRP of 10 days is used.

EXPERIENCE OF TRITIUM INTERNAL MONITORING AT JAERI

(1) Routine Monitoring

In JAERI, routine monitoring for tritium by bioassay was started in 1970. The results of the monitoring are shown in the Table 1.

Table 1 The results of routine monitoring at JAERI

Year	Number Monitored	Number found Contaminated*1
1970	38	0
71	55	4*2
72	52	0
73	61	0
74	105	1*3
75	98	1*2
76	175	0
77	206	0
78	211	0
79	182	0
80	207	0
81	228	1*4
82	254	0
83	284	0
1970-83	2156	7

*1: Significant exposure level is 10 mrem(committed dose)

*2: Van de Graaf accelerator

*3: JRR-2

*4: JRR-3

During the past 14 years, about 2000 measurements were carried out. As a result, only 7 subjects were found out to have significant contamination. For all these subjects, special monitoring was carried out. Doses estimated for these subjects were not large, that is, less than 30 mrem for 6 subjects, and 50 mrem for one.

(2) Special Monitoring

In the past 21 years, as shown in Fig. 2 , the special monitoring carried out for a lot of workers detected 5 cases of contamination. Among these cases, three were related to reactor repair, one was related to an accidental release of tritium gas in Van de Graaff, and the last one was caused by leakage of heavy water in the research reactor. A total of 372 measurements were carried out as special monitoring. As shown in Table 2 , we found about 70 % of the subjects had tritium contamination. However, the estimated committed

doses were very small, less than 250 mrem for most of subjects. For only one subject a dose of over 250 mrem was estimated, which was 380 mrem. This is the highest dose we observed to date.

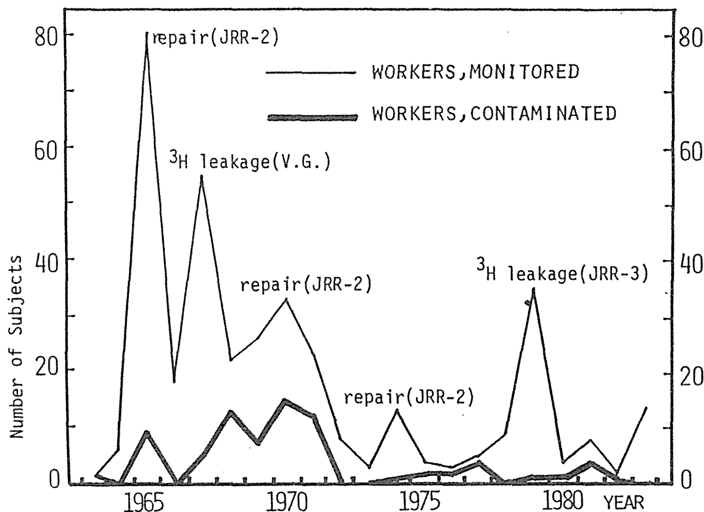


Fig.2 Number of subjects of special monitoring (1963-1983).

Table 2 The results of special monitoring at JAERI

Year	Number of Cases	Number of Subjects	No Contamination	Estimated Committed Dose(mrem)			
				< 10	10-250	250-1000	>1000
1963	1	1	0	0	1	0	0
64	4	6	6	0	0	0	0
65	4	80	22	49	9	0	0
66	8	18	2	16	0	0	0
67	9	55	30	20	5	0	0
68	6	22	0	9	13	0	0
69	7	26	4	15	7	0	0
70	8	33	6	12	14	1	0
71	6	23	5	6	12	0	0
72	3	8	6	2	0	0	0
73	1	3	0	3	0	0	0
74	4	13	5	7	1	0	0
75	3	4	0	2	2	0	0
76	1	3	0	1	2	0	0
77	2	5	0	1	4	0	0
78	2	9	5	4	0	0	0
79	5	35	8	26	1	0	0
80	4	4	3	0	1	0	0
81	3	8	1	3	4	0	0
82	1	2	0	1	1	0	0
83	2	14	0	14	0	0	0
Total	84	372	103(28%)	191(51%)	77(21%)	1	0

(3) Biological Half-life

In the past 21 years, 269 subjects were found to have

tritium (tritiated water) contamination, about 50 of them had relatively high contamination, and the half-life of retention was observed. Careful investigations of their working conditions were carried out, and as the results, 41 subjects were considered as having no further contamination to be noted. All of these subjects were male adults. The observed half-lives are shown in Fig. 3.

The half-lives were in the range of 5-17 days, and an average value is about 10 days. They agreed well with values reported by ICRP (4 to 18 days, a typical value is 10 days). These observed cases are so few that both the seasonal and age variations of the half-life were not obtained from our data.

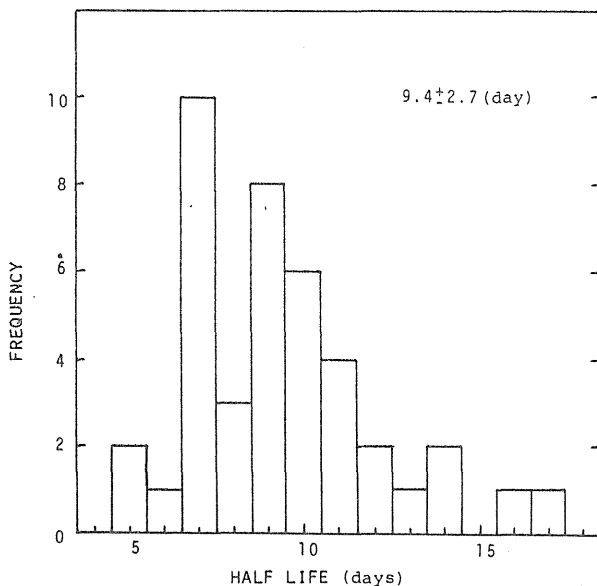


Fig.3 Biological half-lives of tritiated water observed for Japanese male adults.

(4) Comparison of Estimated Doses by Exhaled Air and Urine Analysis

As described before, the exhaled air monitoring of tritium is carried out at the workplace for the workers who

had an accidental intake or who were suspected of tritium intake. The doses evaluated by the two methods, that is by exhaled air monitoring at workplace and by urine analysis, are presented in Fig. 4. In the estimation of dose by exhaled air monitoring at the workplace, the value of the biological half-life is assumed to be 10 days. On the other hand, in the urine analysis, the observed biological half-life can be used in some cases.

As shown in Fig. 4 , doses estimated by exhaled air monitoring at workplace are in good agreement with those by urine analysis.

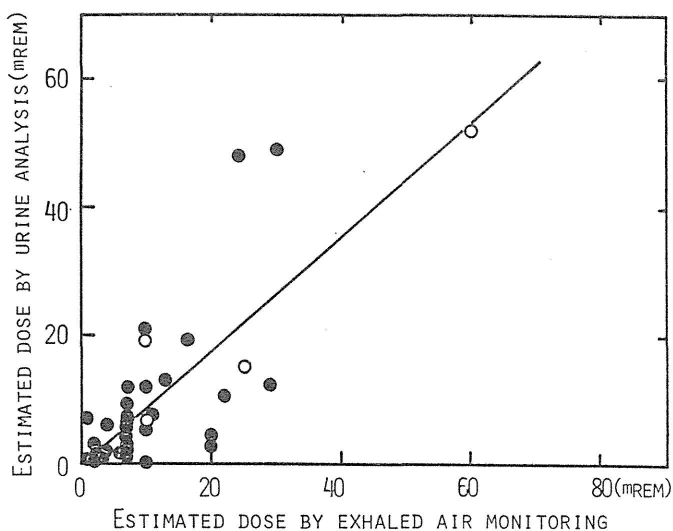


Fig. 4 Comparison of estimated doses obtained by exhaled air monitoring at workplace (●) and urine analysis (o) .

(5) Comparison of External and Internal Exposures of Personnel in Heavy Water Moderated Reactors

The exposure data obtained from 1976 to 1983 of personnel in heavy water moderated reactors are summarized in Table 3.

The external doses for all workers who worked in these facilities were monitored by film badge dosimeter. The film

is developed at an interval of 3 months. The detection limit is 20 mrem per 3 months. The source radiation of external exposure in these facilities was mainly γ-rays.

During this period, several subjects were found to have internal contamination of ^{60}Co or some other nuclides by inhalation. However, estimated doses were very low, and none of them exceeded the significance level (10 mrem). All of the significant exposures observed in these reactors were due to tritium.

As shown in Table 3, the ratio of internal exposure to external exposure was very small.

Table 3 Recent 8 years results of Internal and external exposure at heavy water moderated reactors (1976-1983)

	Number of workers (total)	Exposure	Personal dose		8 years collective dose (person-mrem)	Ratio (internal/external)
			Range (mrem/y)	Mean (mrem/y)		
JRR-2	1892	Internal	0 ~ 52	0.084	160	3.5×10^{-3}
		External	0 ~ 730	24	46×10^{3}	
JRR-3	1838	Internal	0 ~ 15	0.013	23	4.3×10^{-4}
		External	0 ~ 880	29	53×10^{3}	

CONCLUSION

Among about 2000 routine measurements carried out in the recent 14 years, only 7 subjects were found to have a significant internal contamination of tritium. In addition, the results of about 370 special monitorings indicated that internal exposure due to incorporation of tritium was quite low. From these results, it can be concluded that personnel protection for tritium exposure has been carried out very satisfactorily at JAERI.