MASTER

235
3
R-1317
Binn-111

NTIS-25

SP-50

COO-4733-3

**RADIATION PHYSICS, BIOPHYSICS AND
RADIATION BIOLOGY**

Progress Report, October 1, 1979—September 30, 1980

July 1980

Work Performed Under Contract No. AS02-78EV04733

**Radiological Research Laboratory
Department of Radiology
Columbia University
New York, New York**

**TECHNICAL INFORMATION CENTER
UNITED STATES DEPARTMENT OF ENERGY**

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

DISCLAIMER

"This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof."

This report has been reproduced directly from the best available copy.

Available from the National Technical Information Service, U. S. Department of Commerce, Springfield, Virginia 22161.

Price: Paper Copy \$18.00
Microfiche \$3.50

RADIATION PHYSICS, BIOPHYSICS
AND RADIATION BIOLOGY

Progress Report
for Period October 1, 1979 - September 30, 1980

H.H. Rossi, Director
E.J. Hall, Associate Director

Radiological Research Laboratory
Department of Radiology
Columbia University
New York, New York 10032

July, 1980

Prepared For
THE U.S. DEPARTMENT OF ENERGY
UNDER CONTRACT NO. DE-AC02-78-EV04733
(formerly EP-78-S-02-4733)

TABLE OF CONTENTS

INTRODUCTION	6
ACKNOWLEDGEMENTS	8
STAFF	10
 RADIATION PHYSICS	 12
A) <u>Dosimetry</u>	
PD-1 Alteration of field shape and recalibration of x-ray facility P. Goldhagen	13
PD-2 Dosimetry and source design for measurement of the relative biological effectiveness of photons from I-125 P. Goldhagen	14
 B) <u>Microdosimetry</u>	
PM-1 Microdosimetry of x- and γ -rays in a water phantom P. Kliauga (in collaboration with M.N. Varma and J.W. Baum, Brookhaven National Laboratory)	23
PM-2 Microdosimetry at the BEVALAC P. Kliauga and H.H. Rossi (in collaboration with J. Howard, Lawrence Berkeley Laboratory)	27
PM-3 Neutron microdosimetry at RARAF D. Srdoc, L.J. Goodman, S.A. Marino and M. Zaider	42
PM-4 Microdosimetry in the nanometer region M.H. Biavati, H.H. Rossi and P. Goldhagen	61
PM-5 Design and construction of specialized proportional counters for microdosimetry H.H. Rossi, P.J. Kliauga, T. Lukasiewicz and R. Gand	67

PM-6	Microdosimetry of gamma rays: A venture into nanodosimetry region D. Srdoc	81
BIOPHYSICS		89
BP-1	Statistical analysis of cell survival data: A binary approach M. Zaider	90
BP-2	Algorithms for determining the proximity distribution from variance measurements M. Zaider	101
BP-3	Proximity functions for low energy electrons in nitrogen - Application to Iodine-125 M. Zaider	111
BP-4	Saturation effects for sparsely ionizing particles M. Zaider and H.H. Rossi	126
BP-5	Sequential exposures to mixed fields of radiation: A theoretical approach M. Zaider and H.H. Rossi	135
BP-6	Computer activities M. Zaider and P.J. Kliauga	143
RADIATION BIOLOGY		145
RB-1	Oncogenic transformation of mouse C3H/10T $\frac{1}{2}$ cells exposed to 300 kVp x-rays or 35 MeV neutrons R.C. Miller and E.J. Hall (in collaboration with Dr. P. Shapiro, Naval Research Laboratory, Washington, D.C.)	147
RB-2	Oncogenic transformation <i>in vitro</i> produced by misonidazole R.C. Miller and E.J. Hall	155
RB-3	Retinoids and a tumor promoter modify radiation induced transformation but not sister chromatid exchanges in rodent cells R.C. Miller, C.R. Geard, R.S. Osmak, M. Rutledge-Freeman and Carmia Borek	166

RB-4	X-ray induced <i>in vitro</i> neoplastic transformation of human diploid cells C. Borek	188
RB-5	Liver cells in culture: A model for investigating the regulation of retinol-binding protein metabolism C. Borek (in collaboration with Drs. J.E. Smith and D.S. Goodman, Department of Medicine)	197
RB-6	The oxygen enhancement ratio for high energy neutrons E.J. Hall and M. Astor	209
RB-7	Hypoxia mediated drugs as radiosensitizers and cytotoxic agents E.J. Hall, M. Astor, B. Palmer-Alonso and S. Abrams	213
RB-8	<i>In vitro</i> studies with cis-platinum diamide dihydrochloride and exogenous polyamines L. Roizin-Towle and L. Capuano	221
RB-9	The development and decay of thermotolerance M. Freeman	233
RB-10	The sensitivity to x-rays and UV of cis-Plat II sensitive V-79 cells M. Freeman	243
RB-11	Evaluating the interaction of protease inhibitors with DNA in human cells C. Borek (in collaboration with J.E. Cleaver, Laboratory of Radiobiology, University of California, San Francisco)	257
RB-12	Effects of long contact times of misonidazole on attached Chinese hamster V-79 cells C.R. Geard and M. Rutledge-Freeman	271
RB-13	Clastogenic effects of misonidazole on aerated and hypoxic cells <i>in vitro</i> C.R. Geard, L.E. Schwartz and M. Rutledge-Freeman	284
RB-14	Antipain and radiation effects on oncogenic transformation and sister chromatid exchanges in Syrian hamster embryo and mouse C3H/10T $\frac{1}{2}$ cells C.R. Geard, M. Rutledge-Freeman, R.C. Miller and C. Borek	295

RB-15	Interaction of high- and low-LET sublethal damage: effect on the reproductive capacity of cultured human cells P. Furcinitti	306
RB-16	Relative biological effectiveness of photons from I-125 at low dose rates P. Goldhagen, M. Freeman and E.J. Hall	314
RB-17	Sequential exposures with high- and low-LET radiations R.P. Bird	324
RB-18	The associated ion experiment: The effect of proton triads on cell survival R.P. Bird	333
TEACHING		339
INVITED SEMINARS AND PAPERS AT SCIENTIFIC MEETINGS		340
THE COLUMBIA COLLOQUIUM		344
OTHER ACTIVITIES		345
BOOKS		347
BIBLIOGRAPHY		348

INTRODUCTION

The Radiological Research Laboratory fosters a multidisciplinary approach to research in radiation physics and cellular biology. A team of physicists and biologists have been brought together, and a broad-based experimental program is underway ranging from physical measurements of the patterns of energy deposition from x-rays and heavy ions, to the ability of pharmacological agents to induce oncogenic transformations. The diverse experimental approach is carried out within a strong theoretical framework that adds cohesion to the program, allows for a rigorous analysis of the data, functions to interpret the results, and suggest special thrusts in particular areas for the future.

Some of the research is essentially basic and fundamental, while other aspects have immediate pragmatic value; it is often difficult in prospect to determine the category into which a given line of research will lead. For example, the radiation-induced transformation studies have turned out to have substantial significance in radiation protection, while the investigation with hypoxia mediated drugs have an immediate practical impact in radiation therapy. On the other hand, the associated particle experiment addresses the most fundamental questions relating to the spatial separation of sublesions responsible for radiation induced damage.

The year that has passed has been productive. The number and quality of scientific papers presented at National and International meetings, or appearing in refereed Journals, has been maintained at a high level. Members of the laboratory staff are increasingly involved in the affairs of professional societies, on advisory committees to government agencies, or in the grant review system.

After an association lasting more than 20 years Mr. L. J. Goodman has left this Institution for a position with the National Bureau of Standards. We shall remain indebted to him for a great number of vital contributions to our task.

There have been no other changes in the professional staff during the past year. This stability of staff is a result of the fact that the overall size of the laboratory, as well as the blend between physics and biology, is now close to optimal and further expansion of the laboratory at P&S is not envisaged in the immediate future.

The decision to move the Radiological Research Accelerator Facility (RARAF) from the Brookhaven National Laboratory to the Nevis Laboratory of Columbia University should have far-reaching impact in the coming years. It promises to improve significantly our capability to perform research with monoenergetic neutrons and charged particles, because of the closer, more convenient location of the accelerator to our laboratories at P&S.

As in previous years, we are particularly grateful to our principal consultant, Dr. Albrecht Kellerer, who spent three weeks with us during the Spring of 1980. His expertise, enthusiasm, and inquiring mind helped a great deal and materially advanced several of the joint projects between this laboratory and the Institut für Med. Strahlenkunde in Würzburg. We have enjoyed during the past year, in addition, the company of an unusually large number of visiting scientists who presented seminars and advised on our ongoing projects.

To facilitate the exchange of ideas, a Colloquium is being held at approximately monthly intervals, when members of the staff of the laboratory present results of ongoing research. Such occasions are particularly valuable to educate the physicists in the problems of biology and vice versa, and to bring to bear the wide and diverse expertise of the laboratory on the pressing problems of the current research projects.

ACKNOWLEDGEMENTS

Much of the research effort of this laboratory centers around the Radiological Research Accelerator Facility, operated by the Safety and Environmental Division of the Brookhaven National Laboratory. We would not miss this opportunity to thank Dr. V.P. Bond, Mr. C. Meinhold, Dr. N. Rohrig, Mr. S. Marino and the Brookhaven staff who are responsible for the operation of the machine and who will be involved in the move of the machine to the Nevis Laboratory of Columbia University.

Research projects in microdosimetry have been carried out at the BEVALAC facility of the University of California in Berkeley and we are indebted to Drs. J. Howard and H. Grunder for their assistance.

In the area of electron affinic drugs, Roche Products generously supplied substantial amounts of several products, while Dr. Ged Adams, Dr. Martin Brown, and Dr. Parham made available small amounts of newly-synthesized compounds. Bristol Laboratories supplied us at no cost with Bleomycin and a number of other chemotherapy agents were supplied by the Division of Cancer Treatment of the National Cancer Institute.

Most of the instruments and equipment used in the research described in this report were built in the Instrument Shop of the Radiological Research Laboratory. The skills of the shop personnel, under the direction of Mr. Rudolph Gand, have been essential, not only in the construction of apparatus, but also in many important features of design.

A significant portion of the work described in this report was supported by additional funds from other sources. In particular, grants from the National Cancer Institute, DHEW, supported in part the investigations described in the following sections:

CA 12536: PM-2, PM-3, PM-4

BP-5

RB-1, RB-2, RB-3, RB-4, RB-5, RB-7, RB-11, RB-12

RB-13, RB-14, RB-17

CA 15307: BP-2

RB-18

CA 18506: RB-6, RB-8

CA 24232: PD-2

BP-3

RB-9, RB-10, RB-16

CA 23952: RB-1, RB-2

Additional permanent support is furnished by Columbia University. Some of the personnel listed are partly or wholly supported by such other sources.

RESEARCH STAFF

HARALD H. ROSSI, Ph.D., Professor of Radiology (Director)
ERIC J. HALL, D.Phil., D.Sc., Professor of Radiology (Associate Director)
ALBRECHT M. KELLERER, Ph.D., Consultant
MARION H. BIAVATI, Ph.D., Research Associate
RICHARD P. BIRD, Ph.D., Research Associate
CARMIA BOREK, Ph.D., Associate Professor of Pathology (in Radiology)
MICHAEL L. FREEMAN, Ph.D., Research Associate
PAUL FURCINITTI, Ph.D., Research Associate
CHARLES R. GEARD, Ph.D., Assistant Professor
PAUL E. GOLDHAGEN, Ph.D., Research Associate
LEON J. GOODMAN, B.S., Research Associate (resigned April, 1980)
PAUL J. KLIAUGA, Ph.D., Research Associate
RICHARD C. MILLER, Ph.D., Assistant Professor
LAURIE A. ROIZIN-TOWLE, M.S., Graduate Research Assistant
DUSAN SRDOC, Ph.D., Visiting Scientist
MARCO ZAIDER, Ph.D., Research Associate

DESIGN AND INSTRUMENT SHOP

RUDOLPH GAND, Staff Associate (Director)
GARY W. JOHNSON, A.A.S., Design Technologist - Radiology
ADOLF NAPHOLZ, A.A.S., Senior Research Worker/Design

TECHNICAL STAFF

STEVEN ABRAMS, B.A., Technician B
MYLES ASTOR, B.A., Senior Technician
DELIA BROWN, Laboratory Assistant
LOUIS CAPUANO, B.S., Technician B
MARILYN FLYNN, Technician A
MELANIE FREEMAN, M.S., Senior Technician
JOSE GARCIA, Laboratory Assistant
TATIA HARDING, M.S., Technician B
TADEUSZ LUKASIEWICZ, M.S., Technician B
HERBERT O. MASON, Technician B
CAREY JEAN MILLER, Technician A
AUGUSTINUS ONG, M.S., Senior Technician
ROBERT S. OSMAN, B.S., Senior Technician
BARBARA PALMER-ALONSO, B.A., Technician B
ELAINE SIERRA, B.S., Technician B
MARY ZIMMERMAN, B.S., Technician B

SECRETARIAL STAFF

MARY LEE BOONE, B.A., Administrative Assistant
LOUISE S. HO, Senior Secretary
CONSTANCE J. KRAMER, Senior Secretary
SUSAN M. CARLSEN, Senior Secretary
LINDA M. WHITE, M.A., Administrative Aide

RADIATION PHYSICS

A) Dosimetry

- PD-1 Alteration of field shape and recalibration of x-ray facility
P. Goldhagen
- PD-2 Dosimetry and source design for measurement of the relative
biological effectiveness of photons from I-125
P. Goldhagen

B) Microdosimetry

- PM-1 Microdosimetry of x- and γ -rays in a water phantom
P. Kliauga (in collaboration with M.N. Varma and J.W. Baum,
Brookhaven National Laboratory)
- PM-2 Microdosimetry at the BEVALAC
P. Kliauga and H.H. Rossi (in collaboration with J. Howard,
Lawrence Berkeley Laboratory)
- PM-3 Neutron microdosimetry at RARAF
D. Srdoc, L.J. Goodman, S.A. Marino and M. Zaider
- PM-4 Microdosimetry in the nanometer region
M.H. Biavati, H.H. Rossi, and P. Goldhagen
- PM-5 Design and construction of specialized proportional counters
for microdosimetry
H.H. Rossi, P.J. Kliauga, T. Lukasiewicz and R. Gand
- PM-6 Microdosimetry of gamma rays: A venture into nanodosimetry
region
D. Srdoc

A) DOSIMETRY

PD-1 Alteration of field shape and recalibration of x-ray facility

P. Goldhagen

The size and shape of the cone of x-rays emitted by the Siemens therapy machine used for most of the radiobiology experiments performed in this laboratory have been changed. The machine was supplied with a lead collimator which confined the x-rays to a sharply defined square field 25 cm on a side at 100 cm from the axis of the x-ray tube. The exposure rate at the corners of this field was about 10% less than the dose at the center. Concern arose that in cell irradiation experiments some cells were not getting irradiated because occasionally a cell-culture dish was placed partly outside the edge of the field. This possibility has now been eliminated by removing the square lead collimator. The field inside which the exposure rate is within 10% of the central value is now a circle 35 cm in diameter at 100 cm from the tube axis. The area available for irradiating cell culture dishes has thus been increased by about 50%, and the exposure rate decreases gradually rather than abruptly outside this area.

The mean dose rate over the new field has been measured to within $\pm 3\%$ using a Victoreen R-meter at distances of 25, 50, 100, and 150 cm from the tube axis. The results were 7.36, 1.98, .49, and .22 gray/minute respectively.

PD-2 Dosimetry and Source Design for Measurement of the Relative Biological Effectiveness of Photons from ^{125}I

P. Goldhagen

As discussed in Section RB-19 of this Progress Report (1), this laboratory is beginning low dose rate studies of the effects of ^{125}I photons on mammalian cells by comparing the effectiveness of the radiation with that of ^{137}Cs gamma rays in reducing the reproductive viability of exponentially growing cells. This RBE measurement requires that radiation from each of these isotopes, at known dose rates, be available to continuously irradiate cells growing attached to culture dishes in incubators. There are a number of special problems associated with the dosimetry for this measurement which interact with the design of the rest of the experiment. These arise from the low activity of available ^{125}I sources together with the range of dose rates required and from the low energy of ^{125}I photons. A special ionization chamber, designed within the restrictions imposed by these problems, has been built and tested. Actual dose measurements, to be followed immediately by radiobiology, are about to begin.

^{125}I is supplied by the 3M Company encapsulated in 0.8 mm diameter tubes 4.5 mm long with a wall thickness of .05 mm. These "seed" sources are available with 0.55 mCi activity for use in permanent implants and also with "high" activities up to 10 mCi. The fractional variation in activity within a single batch of high-strength seeds increases with nominal activity and is about $\pm 7\%$ for 5 mCi seeds.

To investigate RBE near clinical dose rates, a range of .04 to .4 gray/hour is appropriate (1). Even at such low dose rates, the low and variable

activity of the ^{125}I sources makes it difficult to economically achieve uniform exposure rates over even modest planar areas for cell culture. To achieve the desired range of dose rates, each of three small circular arrays of ^{125}I seeds is used to expose a stack of five 35 mm diameter petri dishes; 3 above the source and 2 below it. This arrangement provides 6 dishes at a low dose rate, 6 at a medium rate, and 3 at a high rate. A computer program has been written which calculates dose rate as a function of radius in a circular plane which is parallel to rings of sources at a given distance, h . Using this program and the height of our petri dishes, we determined that the source array which provides the minimum worst-case nonuniformity for all the dishes in a stack is 14 seeds in a 34 mm diameter circle with an additional seed in the center. With 5 mCi seeds in this array, we get calculated mean dose rates of .41, .14, and .06 gray/hour at $h = 0.6, 1.73, \text{ and } 2.86$ cm with radial variations of $\pm 8\%$, $\pm 8\%$, and $\pm 7\%$. These results include the effect of attenuation in the dish stack, which reduces the dose by about 25% for the top dish. The dose rates will decrease as the isotope decays with a half-life of 60.2 days. Because of the influence of the complicated geometry on the fairly large effects of attenuation and scattering and because of uncertainty in the specific gamma-ray constant for ^{125}I , the uncertainty in these calculated dose rates is about $\pm 20\%$. The dose rate at the growing surface of each dish must therefore be measured for each batch of seeds before cell irradiation is begun.

The ^{137}Cs is arranged in a similar but larger array of 12 sources, 40 mCi each, in a 4.85 cm diameter circle with 3 more sources, 12 mCi each, in a triangle near the center. This array can irradiate eight 35 mm petri dishes (4 above the source, 4 below) at each value of h with radial variations less than $\pm 8\%$ and calculated mean dose rates from .78 down to .12 gray/hr for h .

between 2 and 10.5 cm. These dose rates are about twice those from the ^{125}I sources because we expect an RBE of about 2 from microdosimetric considerations. Photographs of both the ^{137}Cs and ^{125}I irradiation fixtures can be found in section RB-19.

^{125}I seed sources emit gamma- and X-rays with energies between 27 and 35.5 keV. The mean energy is 28.33 keV (2). Photons near this energy present a special problem in determining the absorbed dose in cells attached to culture dishes. The absorbed dose at a point is proportional to the mass energy-absorption coefficient, μ_{en}/ρ , in a region of the surrounding medium where electrons are liberated which can reach the point. At low energies, μ_{en}/ρ changes rapidly with the atomic number of the medium, so that at 28 keV, μ_{en}/ρ for polystyrene is about 62% less than it is for tissue (3,4). At the bottom of a cell attached to a commercial plastic petri dish, half of the region contributing electrons is in polystyrene, and half is in the cell, so the bottom of the cell receives about 30% less dose than it would if it were entirely surrounded by tissue or water. R.D. Colvett, before leaving this laboratory, studied the range of electrons from ^{125}I using a heterogeneous variable-pressure ionization chamber. His findings indicate that the effective range of these electrons in tissue normal to the boundary is $2.8 \pm .8 \mu\text{m}$. This is just about equal to the mean thickness of the nuclei of Chinese hamster cells attached to a commercial plastic petri dish. Such nuclei, exposed to ^{125}I , would experience a 30% variation in dose from top to bottom, and the mean does in each would depend on its exact thickness.

To reduce interface dose variations, we have made our own petri dishes from polyvinylidene fluoride (PVDF) which has a μ_{en}/ρ 1.09 times that of ICRU muscle tissue at 28 keV. The mean dose in cell exposed to ^{125}I while growing on PVDF is therefore about 2% higher than for a cell in suspension, with spatial variations of +3% and -2%. Our PVDF petri dishes have a 0.5 mm thick bottom,

a 35 mm diameter growing surface, and a height of 8.6 mm. They accept standard polystyrene 35 mm petri dish covers. The dishes were compression molded from pellets of Kreha KF 1000 PVDF at a temperature between 185 and 192°C with a pressure of about 6000 lbs/in² (4.1×10^4 kPa). Their low profile, 77% of the height of commercial 35 mm dishes, enabled us to stack them closer together to achieve the range of dose rates from ¹²⁵I mentioned earlier.

An ideal ionization chamber to measure the dose rates from ¹²⁵I absorbed in our cells would be one of our PVDF dishes with a conducting growth surface as the lower electrode, culture medium as the upper electrode, and a 3 µm gap filled with tissue-equivalent gas with a specific gravity of 1. Such a "chamber", whether irradiated from above or below, would measure exactly the radiation field experienced by the cells, including all absorption and scattering effects. The ionization chamber we have built is a practical approximation of such an ideal chamber. The approximation is simplified by the near equality of μ_{en}/ρ for tissue and air near 28 keV. Our chamber is made mostly of air-equivalent conducting plastic, which is 78.41% PVDF, 20.75% carbon, and 0.84% silicon dioxide. The external dimensions, bottom thickness and wall thickness are the same as for one of our petri dishes. Instead of having cells covered with 2.14 mm of culture medium, the chamber has a 1.1 mm air gap covered with a 1.38 mm thick collecting-electrode sandwich which attenuates 27 keV photons the same amount as the layer of medium would. The chamber is even fitted with a polystyrene lid (which is graphite-coated for electrical shielding). A photograph of the ionization chamber and a PVDF dish is shown in Fig. PD-2-1.

An enlarged cross-sectional diagram of part of the petri dish ionization chamber near the edge where the electrical connections are made is shown in Fig. PD-2-2. All electrical leads are provided by a low-noise triaxial cable. Conducting epoxy was used to make electrical contacts to the air-

Figure PD-2-1: Petri-dish ionization chamber and polyvinylidene fluoride petri dish.

Figure PD-2-2: Cross section of petri-dish ionization chamber near the edge where the electrical connections are made.

equivalent plastic electrodes. The alternating layers of conducting and insulating plastic were bonded together with cyanoacrylate adhesive. A 3.2 mm wide guard ring around the collector is formed by a 0.1 mm wide circular groove which was machined through the 0.25 mm thick conducting plastic and slightly into the insulator above. Mylar is used for this critical insulator separating the collector from ground because its volume resistivity is almost 10^4 times that of PVDF. The guard ring is narrow to avoid losing too much collecting area near the edge, where the dose rate may be 8% different from the mean.

The guard ring is too narrow to allow the effective volume of the chamber to be accurately determined by electrical measurements, so the chamber must be calibrated in a known radiation field. This will be done using ^{137}Cs source at Brookhaven National Laboratory which has been calibrated by the National Bureau of Standards. Calibrating with ^{137}Cs allows us to accurately measure the dose rates from the two radiations we are comparing using one ionization chamber. When the chamber or the cell culture dishes are being exposed to ^{137}Cs gamma rays ($E_\gamma = 661.6 \text{ keV}$), they are placed on 1.75 mm thick acrylic plastic shelves to provide a sufficient buildup layer to reach charged particle equilibrium.

The current in the ionization chamber is measured using a Keithly 610 B electrometer as an amplifier with its output fed into a voltage-to-frequency converter. Pulses from the V to F converter are counted for 100 seconds by an electronically-timed counter to provide a digital readout of average current. The electrometer is used in its "fast" feedback mode which maintains the collecting electrode at ground potential.

Figure PD- 2 -3 shows a plot of current vs. collecting voltage taken with the center of the air gap of the chamber 2 cm above our ^{137}Cs source array.

Figure PD-2-3: Graph of current vs. collecting voltage for petri-dish ionization chamber 2 cm above ^{137}Cs source assay.

The plus and minus symbols refer to the sign of the ions collected. The near equality of the positive and negative currents shown in the figure is the result of cancellation of several directional effects at this position and allows both curves to be shown on the same figure. Current measurements will be taken using both polarities and the average used to determine dose rate. Fluctuations in the current readings are less than .01 picoampere so that even for the lowest dose rates that will be measured, calibration errors and uncertainty in the values of μ_{en}/ρ , about 5% (4), will dominate.

REFERENCES

1. Goldhagen, P., Freeman, M., Hall, E.J. Relative biological effectiveness of photons from ^{125}I at low dose rates, USDOE Progress Report C00-4733-3, Section RB-19 (1980).
2. Dillman, L.T., and Von der Lage, F.C. Radionuclide decay schemes and nuclear parameters for use in radiation-dose estimation, Nucl. Med. MIRD pamphlet No. 10 (1975).
3. ICRU Report 17, Radiation Dosimetry: X-rays generated at potentials of 5 to 150 kV, (1970). International Commission on Radiation Units and Measurements, Washington, D.C.
4. Hubbell, J.H. Photon mass attenuation and mass energy-absorption coefficients for H,C,N,O,Ar, and seven mixtures from 0.1 keV to 20 MeV, Radiat. Res. 70:58-81 (1977).

B) MICRODOSIMETRY

PM-1 Microdosimetry of X- and γ - Rays in a Water Phantom

Paul Kliauga, in collaboration with M.N. Varma and J.W. Baum,
Brookhaven National Laboratory

Introduction

A biological system exposed to x-rays or gamma rays experiences a spectrum of radiation which is, in general, a function of depth and may differ considerably from the primary radiation as a result of the contribution from low-energy scattered radiation. This change in spectrum may give rise to a depth-dependence of relative biological effectiveness. The purpose of the measurements described here is to determine to what degree the microscopic distribution of ionization varies as a function of depth in a water phantom when irradiated with a monoenergetic beam of photons.

Experimental

Microdosimetric event distributions were determined in air at these primary photon energies (60, 660, and 1250 keV) and at depths of 2, 5, and 10 cm in a water phantom (30 cm x 30 cm x 30 cm). The 660 and 1250 keV photon energies were from free air sources of ^{137}Cs and ^{60}Co at the BNL calibration facility. For 60 keV a 300 mCi ^{241}Am source was used.

Two counters were used for measurement of the ionization spectra: 1) a conventional walled, Rossi-type counter which was used as the primary counter for all measurements, and 2) a cylindrical "wall-less" type counter used to check the degree of wall-effect distortion by the primary counter. This cylindrical counter, 1/16 inch in diameter, is described in paper PM-5 of this report.

Results

This report summarizes some of the data, taken at 1 μm simulated site diameter. Complete results, for diameters of 0.5, 1.0, and 2.0 μm will be reported at the

The frequency mean ($\bar{y}_F$) and dose mean ($\bar{y}_D$) of the lineal energy density have been calculated from the measurements. This experiment attempts to measure relatively small differences in these quantities. In order to minimize statistical uncertainties, each of the mean values reported is the mean of several determinations, varying from five to fifteen depending on the statistical spread. The statistical uncertainty associated with the values reported is $\sim 1.5\%$ in $\bar{y}_D$ and $\sim 4\%$ in $\bar{y}_F$.

Ratios $R_F = \bar{y}_F$ (water) / $\bar{y}_F$ (air) and $R_D = \bar{y}_D$ (water) / $\bar{y}_D$ (air) are calculated for each depth in the phantom. Table I and Table II summarize the results for a $1 \mu\text{m}$ site diameter.

In Table I, the ratios of $\bar{y}_F$ for the walled and wall-less counters are seen to vary from 1.18 at 60 keV to 1.31 at 1250 keV, while the same ratio for $\bar{y}_D$ varies little. The values are in agreement with other data reported in the literature (1) and the wall-effect is of the magnitude predicted by Kellerer (2).

In Table II, the values of $\bar{y}_F$, $\bar{y}_D$ and the ratios R_F and R_D are given as a function of depth in the water phantom for the wall-less counter, simulating a $1 \mu\text{m}$ diameter x $2 \mu\text{m}$ height cylinder. Only phantom energies of 660 keV and 1250 keV are recorded, since the ratios $R_F = R_D = 1.00$ within experimental error at 60 keV. For 660 keV photons, the maximum increase in the mean values was $\sim 22\%$ at 10 cm depth, whereas for 1250 keV photons, the maximum was $\sim 16\%$. The larger increase in the case of 660 keV photons compared with 1250 keV photons can be attributed to the increased Compton scattering cross section at 660 keV. These ratios would presumably be even higher at ~ 250 keV, where the Compton scattering cross section is larger. Since $\bar{y}_D$ is increased by only $\sim 22\%$, large changes in RBE are not expected as a function of depth in biological systems. A full report on this work will be made at the 7th Symposium on Microdosimetry by one of us (M.N. Varma).

References

1. P. Kliuga and R. Dvorak, Rad. Res. 73: 1 (1978).
2. A. Kellerer, Rad. Res. 47: 377 (1971).

TABLE 1

Values of $\bar{y}_F$ and $\bar{y}_D$ in air for conventional Rossi counter for three photon energies. The numbers in parentheses are the ratios of the corresponding quantity as measured by the wall-less counter ($\bar{y}$ (walled) / $\bar{y}$ (wall-less)). All measurements at 1 μ m simulated diameter.

Photon Energy	Mean y for Walled Counter	
	$\bar{y}_F$	$\bar{y}_D$
60	1.34 (1.18)	3.99 (1.14)
660	0.494 (1.25)	2.25 (1.19)
1250	0.390 (1.31)	1.81 (1.12)

TABLE II

$\bar{y}_F$, $\bar{y}_D$, and the ratios $R_F = \bar{y}_F \text{ (air)} / \bar{y}_F \text{ (water)}$ and $R_D = \bar{y}_D \text{ (water)}$ as a function of depth in a water phantom. All measurements reported for the wall-less counter, at a pressure simulating a 1 μm diameter x 2 μm height cylinder.

Depth (cm)	$\bar{y}_F$	$\bar{y}_D$	R_F	R_D
0 660 keV	0.395	1.89	1	1
1250 keV	0.298	1.62	1	1
2 660	0.403	1.95	1.02	1.03
1250	0.307	1.67	1.03	1.03
5 660	0.450	2.17	1.14	1.15
1250	0.328	1.85	1.10	1.14
10 660	0.474	2.31	1.20	1.22
1250	0.334	1.88	1.12	1.16

PM-2 Microdosimetry at the Bevalac

P. Kliauga and H.H. Rossi in collaboration with J. Howard, Lawrence
Berkeley Laboratory

Introduction

A thorough and accurate microdosimetric investigation of high energy heavy ions (HZE) continues to have a high priority in the physics research at this laboratory. The properties of HZE particles have created growing interest as unique tools in radiobiology and radiotherapy. However, the physics, and correspondingly, the microdosimetry of these particles is extremely complex. This complexity combines with some features of the BEVALAC environment to make a thorough microdosimetry study a difficult, but not impossible, undertaking.

Earlier reports (1 - 5) have dealt with theoretical studies of some aspects of HZE particles, with development of counters and equipment, and some preliminary results. These early results, although deficient in some aspects as noted in earlier reports, had already demonstrated that the conventional wall-less counters constructed in this laboratory for neutrons and photons were inadequate for the BEVALAC particles from the point of view of spectrum distortion by the container walls. Another self-contained, semi-portable wall-less counter, dubbed the "heavy ion counter" was constructed for this work and was sent to the Bevalac for use at that facility. We report here the first measurements with that counter and compare these measurements with a similar counter mounted in a much larger enclosure (See Sect. PM-5 of this report). But first it is worthwhile to summarize some of the experimental difficulties associated with Bevalac microdosimetry.

Experimental Problems

1. High energy (δ -ray structure).

The HZE particles produced by the Bevalac for biomedical research have energies typically of the order of ~ 500 MeV/amu. The maximum δ -ray energies produced are a few MeV, which leads to ranges in tissue of the order of one centimeter! Fortunately, such energetic events are improbable, and a realistic estimate of container size required to introduce negligible distortion in the measured spectra must take into account the strongly energy-dependent cross section for δ -ray production. A simple computer-simulation (4) comparing total ionization in a finite field of heavy ions to total ionization in an infinite field of heavy ions, showed that an error of $\sim 10\%$ could be expected for a tube of 30 cm radius at 68 Torr (simulating 1 μm tissue in a 1/4 inch counter) TE gas.

The tank constructed for use at the Bevalac has a nominal radius of 30 cm. However, the limiting factor turns out to be not the tank, but the beam radius, which can reach maximum dimensions ~ 13 cm in radius. This small size of the beam spot leads to incomplete δ -ray equilibrium, which has been verified experimentally, and is discussed below.

2. Beam spatial distribution

In addition to the small beam size, the beam intensity is not uniform over the area of the beam spot, but has a quasi-Gaussian dependence, with rapidly decreasing intensity in the "tail" region. This type of beam distribution leads to spectral distortion because of under-representation of δ -rays from the outer parts of the beam, due to the fall-off of intensity.

The experimental difficulties associated with points 1) and 2) make it impossible to obtain accurate, "broad-beam" spectra at small simulated site sizes ($\sim 1\mu\text{m}$). On the other hand, because of 1) it is difficult to obtain broad-beam spectra at large site sizes (high gas pressures) because of the exceedingly large amount of energy deposited in a proportional counter by a heavy ion.

As a concrete example, at $100\text{ keV}/\mu\text{m}$ ($450\text{ MeV}/\text{amu}$ ^{40}Ar at the "plateau") and $1\mu\text{m}$ path length, the ion produces $\sim 30,000$ ion pairs, corresponding to $\sim 10\text{ mV}$ out of the preamplifier with no gas gain. At $3000\text{ keV}/\mu\text{m}$ (^{40}Ar near the Bragg peak), the ion produces $\sim 300\text{ mV}$ out of the preamplifier. With an $8\mu\text{m}$ site diameter, these figures become 80 mV and 2.4 V . But in order to see the " δ -ray" part of the spectrum, a moderate, at least, gas gain and sensitivity is needed. Thus, it becomes impossible to observe both primary and secondary contributions to the spectra at the same gas gain or with the same preamplifier sensitivity. Furthermore, at these high gas pressures and low gas gain (small field strength) ion recombination is almost certainly a serious problem.

For these reasons, among others, it appears that the most practical method of obtaining accurate microdosimetric spectra of HZE particles is by taking "differential" spectra, i.e., spectra of a narrowly collimated beam at various distances ("impact parameters") from the sensitive site (collecting volume).

3. Beam temporal profile

The Bevalac beam has a spill time of $\sim 0.5\text{ sec}$, with a repetition rate $\sim 6\text{ sec}$. If the intensity distribution in time were "flat", i.e., constant, and if beam intensity were easily and continuously

variable at low intensities, there would be no experimental problem. Unfortunately, neither is true, and spectrum distortion due to "pile-up" is inevitable unless such pile-up is eliminated electronically. Pile-up occurs when two independent events in the proportional counter occur within a time interval less than ~ 3 times the shaping time of the amplifier. Much effort by the Bevalac electronics crew has gone into the design of an adequate and versatile pile-up rejection system, which is now operational and should allow, with some "tuning", operation at optimum intensity levels in order to speed data collection, as well as avoid distortion errors.

4. Environmental problems

The environmental problems referred to in this section are not ones of chemical or biological pollution, but electronic pollution. The typical accelerator environment is electronically an extremely noisy one. In order to be able to investigate the low energy end of a spectrum, a noise cut-off of $\lesssim 100$ eV is desirable. This corresponds to only ~ 3 ion pairs produced in the counter, or, with a gas gain of 1000, 3000 electrons into the preamp, giving a typical preamp output of ~ 1 mV. The open structures of a wall-less counter act as excellent antennae to pick up much of the stray rf emanating from many known and unknown sources. The counter itself must be well shielded, and it has been found useful to AC-couple counter grid structures to ground. But there still exists many problems associated with pick-up in cables, ground loops caused by the long cabling required from cave to experimental area, etc. These kinds of experimental difficulties can usually only be resolved by a

long and tedious process of trial and error, and this was one of our major sources of difficulty on previous runs. The amount of experimental time did not allow for extensive "noise-chasing", especially of intermittent types which often disappear when not so desired.

The Bevalac crew, together with one of us (J. Howard) spent much time and effort in producing an adequately noise-free electronics system. But there still remain some minor intermittent sources. It was our good fortune on the present run that one such source disappeared at run time, and so did not interfere with data taking.

5. Spallation products

Strictly speaking, this is not a category of "experimental problems", but is included here as one aspect of HZE particles which adds to experimental complications in general. This is because of the enormous number of low-Z spallation products produced. As an example, after traversing ~ 4 cm of water, an incident beam of 500 MeV/amu ^{40}Ar will have as many ^4He as primary beam particles (6), and many more protons. These spallation products have approximately the same velocity as the beam particles, and are strongly forward directed. Therefore, they are highly susceptible to the "V" type of wall effect (7). They are responsible for much of the low-LET (small event sizes) observed.

Apparatus

A specially designed counter, dubbed the "heavy ion counter" was constructed for use at the Bevalac. Design details and drawings are given in section PM-5 of this report. This self-contained "wall-less" type counter consists of a 1/4 inch right circular cylindrical counter mounted inside a brass tube with TE plastic end faces. An exactly similar counter was mounted inside of the large aluminum tank constructed for wall-less measurements. A schematic is shown in Fig. 1. The TE plastic disc, B, was placed inside the tank in order to do wall-effect studies.

Results

All experiments performed at our most recent visit to the Bevalac facility were performed with ^{12}C ions, of approximately 474 MeV/amu. Only one gas pressure was used, corresponding to a unit-density site size of 1 μm diameter simulated by the proportional counter.

All electronics were furnished by Lawrence Berkeley Laboratory, and data were stored in the biomed computer for analysis at a later time. An on-line analysis system has not yet been implemented at the biomed facility. Consequently, raw data were taken back to Columbia University and entered by hand into a PDP 11/45. The spectra were taken at two different electronic gains in order to obtain a better resolution at the low energy end, and were included into a single normalized spectrum for analysis.

Spectra

Figure 2. is a Bragg curve of the ^{12}C beam employed in the experiments. Sufficient time was available for only two points as shown, the "plateau" and "peak" regions.

Figure 1: Schematic diagram of experimental layout at Bevalac. Tank is approximately 8 feet long by 2 feet diameter.

- A: Dacron-mylar beam-entrance window.
- B: Tissue-equivalent plastic disc, 1 foot diameter x $\frac{1}{4}$ inch thick. This is supported on delrin runners C, and is positioned along tank length by rod D.
- E: Cylindrical wall-less proportional counter, $\frac{1}{4}$ inch long x $\frac{1}{4}$ inch diameter.
- F: Plastic scintillator strip, $\frac{1}{4}$ inch x 2 inch x $\frac{1}{16}$ inch thick, mounted on conical light pipe, and designed to move perpendicular to the tank and beam axis on a remotely controlled traverse mechanism (not shown).

FILENAME = LF:[225,206]459B.DAT
 AVISHAI BRAGG CURVE 13-MAR-80 16:21:54 BIOLOGY BENCH
 APAR = 12.00 ZPAR = 6.00 BEVATRON EAMU = 474.00
 28/64 PB SCATTERER

Figure 2: Bragg curve of 474 MeV/amu ^{12}Ci , taken with Bevalac instruments

Figures 3a and 3b were spectra taken on the plateau region with the heavy ion counter. Figure 3a is the conventional "frequency distribution" in which $y \cdot f(y)$ is plotted versus y on a log scale. The area under such a curve represents the relative probability of an event within the interval of y considered. Figure 3b the "dose distribution" shows $y \cdot d(y)$ versus y on a log scale, and the area is proportional to dose. Note in 3a the broad peak centered at ~ 1 keV/ μ m, which is often referred to as the " δ -ray peak" for lack of a better name, but which probably contains much contribution from spallation products, as will be evident by comparison with spectra taken at the peak.

Figures 4a and 4b are spectra taken at the same location, but with an identical type of cylindrical wall-less counter mounted in the large aluminum tank. Thus the only difference between Figures 4 and 3 is the environment of the counters. The " δ -ray peak" in Figure 4a still centered at ~ 1 keV/ μ m, is much more prominent, showing the influence of the wall on the smaller counter in suppressing these events. Figures 5a and 5b were spectra taken near the peak of the Bragg curve, ~ 1 mm upstream, with the large tank. The large number of events in the very broad " δ -ray peak" is consistent with an interpretation that many of these are due to spallation products, which will be very numerous at the peak.

Wall-effect measurements

The tissue equivalent plastic-disc, shown as "B" in Figure 1 was used to test for the amount of wall-effect and presence of charged particle equilibrium. Since time was not available to accumulate full spectra, large electronic gain was used to focus on the low-energy part of the spectrum that we call the δ -ray peak. The variable actually measured is the integral of the spectrum from 480 eV to 4.8 keV divided by the number of monitor counts (beam particles) and

Figure 3: Microdosimetric spectra, plateau position, taken with "heavy ion" counters, described in text. (Figure A)

Figure 4: Microdosimetric spectra, plateau position, as Fig. 3, but taken with large tank (Bevalac counter).

Figure 5: Microdosimetric spectra, peak position of Bragg curve, Bevalac counter.

is thus a measure of the number of low-energy events in this interval per beam particle. Figure 6 shows the result. As expected, as the disc is moved further from the counter, the number of low energy events increases (the "wall-effect" decreases). However, instead of leveling off at some point, indicating the minimum size of counter required to be truly wall-less, the variable peaks and decreases again as the disc is moved further. This behavior can be readily explained in terms of the spatial distribution of delta rays. As the separation between counter and disc is increased it becomes less probable that long range δ -rays traverse the counter together with the beam particles. However, these delta rays are scattered out of the beam, and there is no compensation for this loss if the beam diameter is too small. In other words, charged particle equilibrium does not exist, and larger beam diameters are required, as alluded to in the introduction to this report.

This result confirms a similar finding reported in last year's Annual Report (1) but with only four points, using ^{40}Ar ions.

Conclusions

A reliable, practical microdosimetry system is in place at the Bevalac. Only some minor problems remain to begin systematic extensive studies. Broad beam experiments may be carried out if higher pressures are used, so that charged particle equilibrium can exist. This can be verified using the disc experiment. Thus, either larger simulated site sizes must be used, or a smaller counter must be constructed.

The scintillator and photomultiplier tube has been tested with beam, but the necessary electronics to do an impact-parameter type of experiment has not. The future thrust of Bevalac microdosimetry will undoubtedly focus on the

Figure 6: Ratio of the number of events in the energy interval from 480 eV to 4.8 keV divided by number of beam particles as a function of position of tissue-equivalent disc from the counter, taken at plateau position.

differential impact parameter measurements. But some problems, such as proper beam focussing, have still to be worked out.

Among the many helpful people at the Bevalac, we would like to especially thank Jorge Llacer for his many contributions to development of the pile-up rejection system, data acquisition system, and general assistance. His aid was invaluable and much appreciated.

REFERENCES

1. P.J. Kliauga, Y.M.P. Lam, H.H. Rossi, and L.J. Goodman, Annual Report C00-4733-2, 76 (1979).
2. P.J. Kliauga, R.D. Calvett, L.J. Goodman and Y.M.P. Lam, Annual Report C00-4733-1, 61 (1978).
3. P.J. Kliauga, Y.M.P. Lam, D. Colvett and L.J. Goodman, Annual Report C00-3243-6, 34 (1977).
4. P.J. Kliauga and Y.M.P. Lam, Annual Report C00-3243-5, 96 (1976).
5. P.J. Kliauga and H.H. Rossi, Annual Report C00-3243-5, 106 (1976).
6. R. Silberberg, Private Communication.
7. A.M. Kellerer, Radiat. Res. 47, 377 (1971).

Dusan Srdoc, Leon J. Goodman, Stephen A. Marino and Marco Zaider

Measurements of energy deposition spectra due to monoenergetic neutrons have been continued at RARAF. Previous measurements, briefly reported in Ref. 1, dealt primarily with the influence of the physical and constructional features of proportional counters on measured spectra. Spherical and cylindrical counters of wall-less and solid-wall design were irradiated with 0.44 MeV neutrons and the spectra obtained were carefully examined. The experience gathered during these measurements, as well as the equipment and computer programs, helped us substantially to start a comprehensive program of measurements over a wide range of neutron energies and simulated site sizes. Monoenergetic neutron beams of 0.22, 0.44, 1.5, 6.0 and 14 MeV were used to irradiate spherical wall-less counters simulating tissue volumes having diameters of 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μm .

The final processing of the neutron spectra required a set of gamma-ray spectra obtained by using the same counter at the same gas pressure, and a gamma-ray source, as explained later in this report. Thus, about 160 spectra, including the electronics and proportional counter calibrations and the neutron and gamma-ray spectra, have been obtained and stored on magnetic tapes for further use and processing. Further processing of stored data may introduce minor changes in the final values of $\bar{y}_D$, usually of the order of a few percent. Because $\bar{y}_F$ is quite sensitive to the gamma-ray contamination of the neutron beam, only fully processed data will be presented in this report, as far as this value is concerned.

Experimental Procedure

The experimental technique follows generally that described in Ref. 1. Essentially, the apparatus consists of a Rossi-type spherical wall-less counter coupled to a preamplifier followed by a logarithmic amplifier (both amplifiers

designed by Dr. V. Radeka of the Instrumentation Division, Brookhaven National Laboratory), and the gas lines. There are several important additions to the previously adopted methods, which deal with the calibration technique and the counter operation. In order to check the proportional counter response to very small or very large numbers of ion pairs, produced by energy depositions, two independent calibration sources were applied. The miniature soft x-ray tube emitting the 1.49 keV Al K-line photons served to check the low energy deposition region, while a finely collimated ^{244}Cm alpha source was used for calibrations at high energies.

A 14 MeV neutron microdosimetry spectrum corresponding to 1 μm tissue site size spans more than 3 decades in energy range. It was essential to measure two widely separated calibration points within these energy limits in order to check the linearity of the counter response. The measurements have shown that the voltage between the counter helix and the sphere is quite critical to maintain the correct proportionality in such an unusually wide energy range. The generally accepted constant voltage ratio may lead to serious distortions at the high-energy end of a spectrum. Too low a voltage difference causes loss of charges due to the columnar recombination in a densely packed heavy-recoil track, whereas too high a voltage difference causes a low, but perceptible gas multiplication at the surface of the relatively thin helix wire, leading to an overestimation of the deposited energy. Both phenomena are shown in Table 1 where the calibration energies are compared with the experimental data. A third calibration source was introduced at high counter pressures, where a complete absorption of 5.9 keV photons originating from an ^{55}Fe source takes place within the spherical volume. By measuring three calibration points, which should fall on a straight line if the proportional counter and the associated electronics response are really proportional to the amount of the input charge, one can have confidence in the experimental data obtained under

Table 1

Spherical wall-less counter response to ^{244}Cm alpha-particle calibration source at various charge collecting voltage differences, ΔV

Site size (μm)	1 ΔV (S-H) volts	2 E_{α} (calc) keV/ μm	3 E_{α} (exp) keV/ μm	4 $\Delta E/E \times 100$ %	5 Slope, % per 25 V
8	50	718.4	503.9	- 29.8	
8	100	718.4	609.7	- 15.1	10.5
8	125	718.4	639.1	- 11.0	4.8
8	150	718.4	659.5	- 8.6	3.2
8	175	718.4	681.9	- 5.1*	3.4
8	200	718.4	669.5	- 6.8*	- 1.8
8	225	718.4	703.8	- 2.0	5.1
8	250	718.4	721.0	+ 0.36	2.4
8	275	718.4	762.2	+ 6.8	5.7
8	300	718.4	730.3	+ 1.7	- 4.2
8	325	718.4	781.9	+ 8.8	7.1
4	225	359.2	335.1	- 6.7*	
2	225	179.6	170.8	- 4.8*	
1	225	89.8	84.6	- 5.7*	
1	210	89.8	83.1	- 7.4*	
1	135	89.8	81.8	- 8.9	
0.5	100	49.9	39.3	- 12.4	

Mean departure from the calculated E_{α} , using

225 > ΔV > 175V (middle of the plateau)(x): $6.1 \pm 1\%$

1. Voltage difference between the sphere and the helix

2. Based on $\text{LET}_{\alpha} = 89.8 \text{ keV}/\mu\text{m}$ for ^{244}Cm .

3. Energy scale calibrated with Al x-ray source ($E_{K\alpha, \text{Al}} = 1.495 \text{ keV}$).

$$4. \Delta E/E \times 100 = \frac{E_{\alpha(\text{exp})} - E_{\alpha(\text{calc})}}{E_{\alpha(\text{calc})}} \times 100$$

5. Slope of saturation curve, $(E_{\alpha(\text{exp})2} - E_{\alpha(\text{exp})1})/E_{\alpha(\text{exp})1} \times 100$ per 25V

the conditions of an extremely wide energy range. Our practice was to check the electronics as well as the counter response before each measurement of the neutron ionization spectrum.

A close inspection of data presented in Table 1 reveals another phenomenon which is important, particularly in high-energy neutron microdosimetry. Whereas the agreement between the nominal and the experimental calibration data is very good for the Al x-ray and the ^{55}Fe sources, a systematic discrepancy varying from -4 to -7% exists between the photoelectron and the alpha-particle sources. This applies to the region where no distortion of the experimental values takes place due to the already mentioned factors. There are two factors which contribute to this discrepancy: 1) The exact volume and the shape of the charge collecting sphere is not very well defined in a wall-less counter, and 2) The W-value for alpha particles is 6 to 7% higher than the W-value for electrons for most gases and gas mixtures. Provided that in our case the first factor is not very significant, i.e., that the physical and the actual collecting volumes are very close, then the differences listed in Table 1, column 5 can be completely explained by the difference in the W-values between the electrons and the alpha particles. This fact complicates the calculation of the lineal energy scale, especially for high energy neutrons where one encounters at least four overlapping sections (electrons, protons, alpha-particles, and heavy recoils), each having a slightly different W-value.

Measurements and Processing of Spectra

Two spherical, wall-less Rossi-type counters have been used throughout the measurements. The "1 inch" counter, used in many previous measurements (2) is described in detail in Ref. 3, whereas the " $\frac{1}{4}$ inch" counter is essentially of the same design, scaled down by factor of 4. There are, however, several distinct features pertinent to the " $\frac{1}{4}$ inch" counter: 1) as opposed to the "1 inch" counter,

it is enclosed in a thin aluminum shell; 2) it has an inner layer, made of tissue-equivalent plastic which can be changed thus allowing a study of the influence of the plastic layer thickness on the spectra; 3) an additional layer of tissue-equivalent plastic can be added outside the aluminum shell. The small physical size has two distinct advantages when compared to the larger model. A four-fold increase of the pressure for the same site size adds to the counter operational stability and it allows stable operation at much smaller site sizes.

We started measurements with the "¼ inch" counter and replaced it with the "1 inch" counter when the small one developed a gas leak. No difference in mean values $\bar{y}_F$ or $\bar{y}_D$ or in shapes of spectra has been observed using either counter for a range of neutron energies or site sizes. Also, there was no change in spectrum when an additional 3 mm outside layer of tissue-equivalent plastic was placed on the aluminum shell. Therefore, subsequent measurements were made without any outside layer of TE plastic. However, the thickness of the inner layer of TE plastic is very critical at high neutron energies. A 0.25 mm layer, used for measurements up to 4 MeV neutrons, had to be replaced by a 3 mm thick layer for higher neutron energies in order to obtain particle equilibrium. Figure 1 shows the difference between the equilibrium and non-equilibrium spectra for 14 MeV neutrons, 1 μ m site size. Note that the mean values $\bar{y}_F$ and $\bar{y}_D$ are extremely sensitive to the non-equilibrium condition.

A careful alignment and positioning of the counter with respect to the neutron production target was performed before each measurement. Table 2 gives details of the experimental arrangements. The counter was usually vacuum pumped overnight, then checked for leakage and filled with propane based tissue-equivalent gas to the pressure required to simulate a certain tissue size. In order to achieve gaseous equilibrium, i.e., to prevent absorption by or outgassing from the plastic lining from changing the counter gain, a steady flow of TE gas was

Figure 1: $d(y)$ vs $\log y$ spectra, influence of inside layer thickness; 14 MeV neutrons, $1 \mu\text{m}$ site size; wall-less spherical counter, $\frac{1}{4}$ inch in diameter; Curve 1: 0.25 mm TE plastic layer inside the aluminum shell; Curve 2: 3 mm TE plastic layer. Both curves are normalized.

Figure 2: Processing of neutron $d(y)$ vs $\log y$ spectra. 0.22 MeV neutrons, $1 \mu\text{m}$ site size. Curve 1: Experimental spectrum, neutrons and gamma rays. Curve 2: ^{60}Co gamma-rays $d(y)$ vs $\log y$ spectra, $1 \mu\text{m}$. Curve 3: Net neutron spectrum, obtained by subtracting the gamma-ray spectrum from the experimental spectrum.

Table 2
Neutron Source Data and Counter Geometry in Respect
to Target and Beam Line

Neutron Energy (MeV)	Reaction	Counter position	
		Distance to target (cm)	Angle from ion beam (deg)
0.22	T(p,n)	11.5	100
0.44	T(p,n)	11.5	120
1.5	T(p,n)	10.4	0
6	D(d,n)	11.5	0
14	T(d,n)	12	100

Table 3
Dose Mean Lineal Energy $\bar{y}_D$ as a Function of Neutron
Energy and Site Size as Measured by the Rossi-type
Wall-less Spherical Counter Filled with Propane-
based Tissue-equivalent Gas

Neutron Energy (MeV)	Spherical site size (μm)					
	0.25	0.5	1.0	2.0	4.0	8.0
0.22	—	74.5	69.3	—	—	—
0.44	80.5	76.5	73.7	64.1	45.7	29.2
1.5	67.6	64.4	64.8	57.7	47.5	34.3
6	60.1	57.5	57.0	48.3	37.9	26.7
14	—	89.0	89.6	—	67.5	49.6

maintained for awhile. This was achieved by means of a pressure and flow regulating device (Granville-Philips). It was found, however, that a closed system was very reliable in terms of the stability and resolution of the calibration peaks. Most of the data were, therefore, obtained in the closed system mode. The electronics was checked for overall gain stability and logarithmic response using a precision pulser. Four equally spaced peaks, resulting from setting the precision pulser to four predetermined pulse heights, each differing by a factor of 10, were checked for position (channel number) and spacing (channels per decade). Next an energy calibration was performed using the Al K-line and the ^{244}Cm alpha source. The system was then ready for data acquisition. All relevant data, such as the calibration energy and corresponding mean channel number, the gas pressure, etc., were recorded on the computer.

The data processing deserves special attention in this report. Besides the technical problem of coping with a large number of spectra, the following operations had to be performed:

- 1) to display or plot the $d(y)$ spectrum and calculate the mean value;
- 2) to convert the $d(y)$ spectrum into the $yd(y)$ spectrum and calculate the mean value;
- 3) to shift the spectrum in either direction along the (logarithmic) abscissa, and to match or compare two spectra taken at different gains;
- 4) to combine sections from different spectra (usually taken at different gains);
- 5) to normalize the spectrum or to multiply the ordinate by a factor in order to match two spectra;
- 6) to extrapolate the low energy end of a spectrum;
- 7) to subtract a gamma-ray spectrum from a neutron spectrum.

While most of the above-mentioned computer operations helped to process the data efficiently, the last mentioned operation 7) served to eliminate the gamma-ray contamination from the neutron spectra. This procedure is shown in Fig. 2.

Here the $d(y)$ vs $\log y$ spectrum is plotted for 0.22 MeV neutrons and 1 μm site size. The ^{60}Co $d(y)$ vs $\log y$ spectrum for 1 μm site size is plotted on the same graph, matching the peak height on the left side of the neutron spectrum. It is evident from the shapes of the overlapping section of both spectra that the energy deposition below $\sim 1 \text{ keV}/\mu\text{m}$ is entirely due to gamma-rays. The region between 1 and 10 $\text{keV}/\mu\text{m}$ consists of a mixture of a high energy tail of gamma-ray depositions and a low energy tail of short, truncated proton segments within the sphere. Above 10 $\text{keV}/\mu\text{m}$ the spectrum is entirely composed of recoil protons. It should be pointed out that the gamma-ray energy depositions which contaminate the neutron beam are obviously not identical with a ^{60}Co gamma-ray spectrum. However, the difference in microdosimetry spectra for gamma rays having a wide range of energies, such as ^{137}Cs ($E = 0.66 \text{ MeV}$), ^{60}Co ($E_1 = 1.2 \text{ MeV}$, $E_2 = 1.33 \text{ MeV}$) and ^{24}Na ($E = 2.76 \text{ MeV}$) is relatively small, thus justifying the subsequent step consisting of subtraction of ^{60}Co gamma-ray spectrum from the neutron and gamma-ray spectrum shown in Fig. 2. The resulting spectrum represents the net neutron contribution to the energy deposition spectrum. The mean value $\bar{y}_F$ changes drastically after subtraction of the gamma-ray contamination. In the case shown in Fig. 2 the $\bar{y}_F$ value increased from 12.2 $\text{keV}/\mu\text{m}$ to 37.1 $\text{keV}/\mu\text{m}$, while the $\bar{y}_D$ value has changed from 63.9 $\text{keV}/\mu\text{m}$ before subtraction of gamma-rays to 69.3 after subtraction. The difference decreases as the neutron energy increases.

When evaluating microdosimetric data of the type presented in Fig. 2, or the mean values $\bar{y}_F$ and $\bar{y}_D$, one has to bear in mind the ultimate use of these data. For radiobiological purposes the uncorrected values may be more relevant, since the irradiated samples will absorb both the gamma-rays and the neutron induced radiation (recoil protons). On the other hand, for comparison with calculated values of $\bar{y}_F$ and $\bar{y}_D$, as well as for radiation physics purposes, it is better to have a clear cut picture of the contribution of each type of radiation to the total

absorbed dose and their respective microdosimetric spectra.

Results

The most concise way to present the microdosimetric data is to tabulate mean values $\bar{y}_F$ and $\bar{y}_D$ for the entire range of measured neutron energies and site sizes, as shown in Table 3. Additional information can be obtained from the $d(y)$ vs $\log y$ and $y d(y)$ vs $\log y$ spectra. Several examples of such spectra, taken from a large number of measured spectra, are shown in the following figures. Figure 3 shows the variation of the shape of the $y d(y)$ vs $\log y$ spectrum for 0.44 MeV neutrons with site size as the parameter ranging from 1 to 8 μm . As opposed to the smaller site sizes shown for clarity on a separate graph (Fig. 4), which have almost identical shapes, the spectra above 1 μm shift to lower lineal values as the site size increases. This shift is accompanied by a broadening of the distributions. The physical picture is relatively simple and straightforward. The proton recoil flux, having a spectrum of approximately rectangular shape is intercepted by spheres of various dimensions. At a sphere diameter equivalent to 1 μm of tissue or below, most of the recoil protons cross the volume, resulting in a relatively sharp peak in the $y d(y)$ representation. As the sphere diameter increases above 1 μm , an increasing fraction of recoil protons deposit their entire energy within the sphere. Further increases of the sphere diameter are not accompanied by more energy deposition and therefore the whole spectrum is shifted toward lower y -values. The broadening of the spectrum at higher site size is caused by an additional term in the total variance, reflecting the shape of the initial recoil proton spectrum.

The variation of the shape of the $y d(y)$ vs $\log y$ spectrum with neutron energy for a site size of 1 μm is shown in the next figures. Figs. 5 and 6 show the change of the spectrum shape for neutron energies equal to 0.22, 0.44, 1.5, 6 and 14 MeV. The 0.22, 0.44 and 1.5 MeV spectra have a sharp proton cutoff at approximately 145 keV/ μm . As opposed to the lower energy neutrons (1.0 MeV and below)

Figure 3: $y_d(y)$ vs $\log y$ spectra, 0.44 MeV neutrons. Variation of spectra shape and position with increasing site size from 1 to 8 μ .

Figure 4: $y_d(y)$ vs $\log y$ spectra, 0.44 MeV neutrons. The shape and position of spectra representing tissue spheres of 1, 0.5 and 0.25 μ in diameter do not change appreciably. Their respective mean values $\bar{y}_D$ change by several percent (see Table 3).

Figure 5: Comparison of $yd(y)$ vs $\log y$ spectra, 1 μm site size, 0.44, 1.5 and 6 MeV neutrons. For complete description see text.

Figure 6: Comparison of $yd(y)$ vs $\log y$ spectra, 1 μm site size, 0.22, 0.44, 1.5 and 14 MeV neutrons. For complete description see text.

the recoil proton peak is shifted to lower y -values: for 6 MeV neutrons the proton peak lies around 17.5 keV/ μm whereas for 14 MeV neutrons it lies around 9 keV/ μm . On the other hand, neutrons above 1 MeV start producing heavier recoils with sufficient efficiency to be registered on the microdosimetry spectra. A short "tail" belonging to alpha particles is evident between 150 and 300 keV/ μm on the 1.5 MeV spectrum, becoming more prominent as the neutron energy increases (see Fig. 5, 6 MeV neutrons). The high energy tail splits at 14 MeV neutron energy into distinct peaks, belonging to alphas and heavy recoils, as indicated in Fig. 7. The heavy recoils, consisting of carbon and oxygen ions cannot be separated using the present technique.

The variation of the shape of the $y_d(y)$ spectra with site size for 14 MeV neutrons is shown in Figs. 8 and 9. Again, there is no significant difference among 0.25, 0.5, and 1.0 μm site size spectra as demonstrated in Fig. 8. At larger site sizes, i.e., above 1 μm , the general trend is a shift to the left. This means that a fraction of the heavy particles has a range comparable to 1 μm in tissue. The proton contribution to the absorbed dose is steadily increasing with the site size (Fig. 9).

Discussion

Most of the experimental data on neutron microdosimetry have been performed at the Radiological Research Laboratory and published in the Annual Reports. There are two distinct groups of reports: the older ones by M.H. Biavati et al., published from 1964 through 1976 (4,5,6) and the recent ones by Rodgers, Dicello, Gross and Rossi (7,8,9). A summary of the latter reports was presented by Rodgers and Gross at the Fourth Microdosimetry Symposium (1973) pointing out, however, that the data presented were preliminary (2). The disagreement between the two sets of data ranged from 10 to 30% and prompted the comprehensive and systematic

Figure 7: $yd(y)$ vs $\log y$ spectra, $1 \mu\text{m}$ site size, 14 MeV neutrons. Several distinct regions are marked on the spectra, indicating contributions from protons, alpha particles and heavy recoils to the total absorbed dose, which is proportional to the area under the curve in this notation.

Figure 8: 14 MeV neutrons, $yd(y)$ vs $\log y$ spectra. Variation of spectrum shape with site size for 1, 0.5 and $0.25 \mu\text{m}$ sphere diameters.

Figure 9: 14 MeV neutrons, $yd(y)$ vs $\log y$ spectra. Variation of spectrum shape with site size for 1, 4 and 8 μm sphere diameters.

measurement of neutron spectra presented in this report. A comparison of three sets of experimental data obtained at RRL, together with the results of calculations by Caswell and Coyne (10) is made in Table 4. The present data agree well with those of Rodgers and Gross. The calculated $\bar{y}_D$ values (10) as well as those of M.H. Biavati et al., (5) are higher. It should be mentioned that the calculated values shown in Table 4 are for ICRU tissue, whereas the experimental values presented by Rodgers and Gross and in this report were obtained using propane-based tissue-equivalent gas. M.H. Biavati et al., used methane based tissue-equivalent tissue-equivalent gas. Their first reported $\bar{y}_D$ values (4) were calculated assuming the LET for ^{244}Cm alpha particles used for calibration was equal to 77.8 keV/ μm . In the following reports the LET for alphas was recalculated using new data for stopping power. They obtained 87.7 keV/ μm for ^{244}Cm alpha particles; this value was used in their reports from 1965 on. Therefore, a correction must be applied in order to compare all three sets of experimental data. First, the old notation was converted into present notation by multiplying the $\bar{y}_D$ values by 3/2 (Table 4, column 4); next the calibration correction factor equal to 1.127 was applied to the data calculated using the $\text{LET}_\alpha = 77.8$ instead of 87.7 (Table 4, column 5). Again, the present data, the Rodgers and Gross data and the LET_α corrected early data of M.H. Biavati et al., for 14.7 MeV neutrons and 1 μm site are in a surprisingly good agreement, leading to an average value $\bar{y}_D = 89.5$ keV/ μm and less than 1% departure for each individual measurement from the mean value. However, when other site sizes, as well as the 1965 data of M.H. Biavati et al., are compared, the agreement is not nearly as good as in the previous case. The calculated $\bar{y}_D$ values of Caswell and Coyne for ICRU tissue are generally higher than the experimental data by 10 to 25% with the exception of Biavati's 1965 data (5). It should be pointed out, however, that 3 out of 4 experimental sets of data

Table 4

14 MeV Neutrons, $\bar{y}_D$ in keV/μm for Various Site Sizes
Comparison of Experimental Data and Theoretical Calculations

	This Report	Rodgers Gross (2)	Dicello (12)	M.H. Biavati et al. 1964(4) LET=77.8	1964(4) LET=87.7	1965 (5)	Caswell Coyne calc. (10)
Site size:	0.5μ	0.5μ	0.45μ 0.47μ	0.4μ	0.4μ	0.49μ	0.5μ
$\bar{y}_D$ W-less:	89	86.6	91.7	—	—	—	101
Wall:	—	—	94.2	97.8	110	113.2	—
Site size:	1.0μ	1.0μ	0.95μ 1.01μ	1.0μ	1.0μ	0.99μ	1.0μ
$\bar{y}_D$ W-less:	89.6	88.7	94.5	—	—	—	100
Wall:	—	—	94.1	80.1	90.3	103.9	—
Site size:	2.0μ	2.0μ	—	2μ*	2μ*	2μ*	2μ
$\bar{y}_D$ W-less:	—	86.0	—	—	—	—	96.5
Wall:	—	—	—	79	89	97	—
Site size:	4.0μ	4.0μ	—	4μ*	4μ*	4μ*	4μ
$\bar{y}_D$ W-less:	67.5	72.3	—	—	—	—	83.5
Wall:	—	—	—	79	89	84	—
Site size:	8.0μ	8.0μ	—	8μ*	8μ*	8μ*	8μ
$\bar{y}_D$ W-less:	49.6	52.5	—	—	—	—	66
Wall:	—	—	—	74	83	68	—

*Interpolated values

(including early Biavati et al., data (4)), show the same trend, i.e., have a consistently lower $\bar{y}_D$ values than the calculated ones. The discrepancy can be explained at least qualitatively as follows: 1) The experimental values need correction for change of the W-value, as already explained; 2) More precise and complete input data for analytic calculation are required as pointed out by Caswell and Coyne (10); 3) It would be desirable to calculate $\bar{y}_D$ values specifically for tissue-equivalent gases, methane- and propane-based. While the W correction will definitely help to bring the experimental and the calculated data closer, the effect of the remaining two calculations remains to be seen.

The assessment of the so-called wall-effect has been very often the target of both experimental (8,9) and theoretical studies (11). This work was also preceded by a measurement of the wall-effect (1); a more complete analysis will be presented at the forthcoming Microdosimetry Symposium in Oxford, U.K., this year. By comparing published data one can estimate that the magnitude of the wall-effect never exceeded $\sim 10\%$ of the $\bar{y}_D$ value.

Therefore, errors introduced by various counter designs, different calibration techniques, gas gain variation, etc., might in fact exceed the magnitude of the wall-effect.

This report presents only part of the ample experimental data obtained. Further processing and analysis of spectra will undoubtedly expand our knowledge on the problems which were just mentioned. Among other things, calculations of the $\bar{y}_F$ values for clean neutron spectra, which require a considerable amount of computer time have been planned. A collaboration with R.S. Caswell and J.J. Coyne of the National Bureau of Standards is planned in order to improve the theoretical calculations.

References

1. Srdoc, D., Mills, R.E. and Marino, S.A., Microdosimetry measurements at RARAF Annual Report on Research Project C00-4733-2, p. 95, (1979).
2. Rodgers, R.C. and Gross, W. (1974) Microdosimetry of monoenergetic neutrons. Proc. 4th Symp on Microdosimetry, Euratom Document EUR 5122 d-e-f, p. 1027.
3. Rodgers, R.C., Dicello, J.F. and Gross, W. The Biophysical Properties of 3.9 GeV Nitrogen Ions, II. Microdosimetry, Radiat. Res. 54: 12-23 (1973).
4. Biavati, M.H., Rossi, H.H., Hutton, A. and Boer, E. Monoenergetic Neutron Studies - 14.7 MeV. Annual Report on Research Project NYO-2740-1, p. 32-48 (1964).
5. Biavati, M.H., Rossi, H.H. and Boer, E. Monoenergetic Neutrons - 14.7, 5.7, and 3.7 MeV. Annual Report on Research Project NYO-2740-2, p.41-76 (1965).
6. Biavati, M.H., and Boer, E. Monoenergetic Neutrons. Annual Report on Research Project NYO-2740-4, p. 74-80 (1967).
7. Rodgers, R.C., Gross, W., and Rossi, H.H. Event Distributions from Neutrons, a) Monoenergetic neutrons from RARAF. Annual Report on Research Project. NYO-2740-8, p. 38-46 (1971).
8. Rodgers, R.C., Dicello, J.F., and Gross, W. Event Distribution from Monoenergetic Neutrons. Annual Report on Research Project C00-3243-1, p. 52-59 (1972).
9. Rodgers, R.C., Dicello, J.F., and Gross, W. Event Distribution from Monoenergetic Neutrons. Annual Report on Research Project C00-3243-2, p. 87-101 (1973).
10. Caswell, R.S. and Coyne, J.J. Microdosimetric Spectra and parameters of fast neutrons. Fifth Symp on Microdosimetry, Verbania Pallanza (Italy), Sept 22-26 (1975), p. 97-125.
11. Kellerer, A.M., An Assessment of Wall Effects in Microdosimetric Measurements, Radiat. Res. 47: 377-386 (1971).
12. Dicello, J.F., Private communication.

M.H. Biavati, H.H. Rossi and P.E. Goldhagen

Experimental work on the grid avalanche chamber which was designed for dosimetry measurements in the nanometer region has been continued. A Le Croy qVT, 1024 channel analyzer has been incorporated as part of the experimental system. This multichannel analyzer has a charge sensitive input, and total charge can be integrated for time lengths from twenty nanoseconds to one microsecond. Setting the integration time as short as possible (the photomultiplier tube has a rise time of approximately ten nanoseconds) will result in a better signal to noise ratio. A special negative high voltage supply (Vern Keibler) for the grid has been obtained. This supply is particularly useful because it can be set to automatically cut off the high voltage when the current drawn by the grid is greater than 80 nanoamperes. In order to get the best signal to noise ratio it is advantageous to set the grid voltage as near to breakdown as possible. However, once breakdown occurs the maximum voltage attainable decreases, which is a common problem with other detectors as well, for example, proportional counters. The V.K. power supply is specially designed to keep breakdown currents to a minimum. The gas flow system being used, although adequate for preliminary measurements, has several deficiencies. It is very difficult to set predetermined pressures accurately. Also, maintaining low pressures is possible for short time intervals, but there is usually a slow steady drift either upward or downward over a period of a few hours. The parts for a new gas flow system and pressure regulator have been obtained, utilizing an MKS Baratron pressure and flow regulator, but the system has not been designed or assembled.

Provisions will also be made to cool the tube down to the minimum allowable

(0°C) temperature in order to further decrease noise.

Measurements thus far have been made with a Cm^{244} alpha source placed so that alpha particles cross the chamber and are counted in a solid state detector. The spectrum of alpha particles in the solid state detector, taken at minimum pressure (0.2 Torr) is shown in Fig. 1. It is not symmetrical, and the increased width at low energies is due to the source thickness, i.e., some of the alpha particles are degraded in energy before getting out of the source. The actual resolution of the solid state detector is not important because it will only be used to indicate the actual passage of an alpha particle through the sensitive volume. Background and noise measurements in the photomultiplier tube were made by externally rotating the source in a horizontal plane so that the alpha particles impinge on the wall of a lucite collimator and are not able to penetrate the sensitive volume. Measurements were made with the alpha source, at several pressures and positive results (a significant increase in counting rate with alpha source) were obtained at a few grid and screen voltages. A summary of the pressures and voltages at which ions were counted is given in Table 1. Note that the voltage at the screen and the drift voltage across the chamber are not independently variable. There is one negative power supply which provides the voltage for the three screen wires and then a voltage divider determines the drift voltage across the chamber. The voltages are in the same ratio as specified in Annual Report 1978 although the actual value of the screen voltage has been increased. Increasing the screen voltage also increases the drift voltage across the chamber and in the detector region. Measurements at lower screen voltages were made, but no ions were counted at the particular pressures and grid voltages that were used.

Figure 2 shows the background and noise with the grid and screen voltages turned on for three pressures. The maximum grid and screen voltages are set by

CM²⁴⁴ Solid State Detector

Figure 1: Alpha particle spectrum from Cm²⁴⁴ as measured in the solid state detector. Note that counts are plotted on a linear scale.

Figure 2: Background and noise as measured with the photomultiplier tube with the grid and screen voltages turned on. Note that counts are plotted on a logarithmic scale.

Figure 3: Positive ions from Cm²⁴⁴ alpha source as measured in photomultiplier tube at the three gas pressures indicated.

comparing the spectrum without source, but with the grid and screen voltages alternately turned on and off. It has been found that raising the grid and screen voltages beyond a certain level increases the background counts significantly, see Fig. 2, $P = 0.08$ Torr, even before breakdown is reached.

Since it was necessary to determine if light was coming from alpha particles producing ions directly in the chamber volume and reaching the photomultiplier tube by multiple reflections at the various surfaces, measurements were made at several pressures with the source in and out, at zero grid, screen and drift voltage (See Table 1). No significant difference (beyond statistics) in counting rate was obtained, indicating that no light is produced in the gas in the chamber volume. The light detected by the photomultiplier tube must therefore be produced directly in the detector region (See. Fig. 2) around the high voltage grid or in the scintillator by secondary electrons emitted by the grid. It is possible to determine which of the two previous effects occur by replacing the plastic scintillator with a non-scintillating piece of plastic, but this has not yet been done. As far as the successful operation of the chamber is concerned it makes no difference which effect causes the light. The efficiency of the effect, i.e., the amount of light produced per ion and whether or not this is a constant (and never zero), is of greater significance and will have to be studied in detail.

For very low pressures, representing track lengths less than one nanometer (0.045 Torr) at most one ion will be produced by each alpha particle as it traverses the sensitive volume of the chamber. As the pressure is decreased still further, practically no ions will be produced. Therefore, at really low pressures the difference between "source in" and "source out" measurements should be negligible, which it is. The total counting rate should then increase as pressure increases which has been verified (See Fig. 3). The maximum pressure at which

data was taken is 0.08 Torr using the same voltages throughout from 0.02 Torr to 0.08 Torr. At 0.092 Torr the grid voltage had to be dropped due to intermittent breakdown, resulting in a shift in calibration and therefore the counting rates are not comparable with the lower pressures.

TABLE 1

Pressure	Grid V	Screen V
0.02	-1500	-500
0.045	-1500	-500
0.054	-1500	-500
0.08	-1500	-500
0.092	-1200	-500
0.02	0	0
0.054	0	0

PM-5 Design and Construction of Specialized Proportional Counters
for Microdosimetry

Harald H. Rossi, Paul J. Kliauga, Tadeusz Lukasiewicz, and Rudolph Gand

Introduction

A number of proportional counters incorporating specialized designs have been built during the last two years. In most cases these have not been described in detail elsewhere. It is the intention of this report to furnish reasonably detailed drawings of these counters and to briefly describe the outstanding or unique features of each counter in the context of its intended use or application. The drawings are not intended to be "shop" drawings for construction purposes, but are intended to provide a clear perception of the construction and design features peculiar to each counter, and how particular design ideas were realized in practice.

All of these counters were created for specific purposes. The "naming" of the counter is for convenience only, and is entirely arbitrary and capricious.

Bevalac Counter

This counter (Fig. 1) was designed for use at the Bevalac facility for heavy ion microdosimetry, as described elsewhere in this report. It is mounted in the large aluminium tank constructed for this purpose. It was intended to be a rugged, simple, easy-to-use type of counter (it has already been transported from Berkeley to New York several times). For these reasons, the most basic type of counter geometry was chosen. The collecting volume is a $\frac{1}{4}$ inch right circular cylinder, bounded by the helix along the diameter, and the "field tubes" which help to shape the field geometry, along the axis. Its resolution is not as good as some of the spherical wall-less types, but is adequate for its intended use. It is, of course, operated as a "wall-less" type counter, with the walls of the tank being the only limiting geometry except for the narrow ($\frac{1}{4}$ inch thick) supporting ring.

Figure 1: Bevalac counter

However, as noted elsewhere, it turns out that the practical beam size at the Bevalac is more limiting. Gas amplification is adequate.

Heavy Ion Counter

This is quite similar in design to the Bevalac counter described above, and is shown in Fig. 2. The same basic simple cylindrical geometry has been used, except the counter is mounted inside of a brass tube with tissue-equivalent plastic end caps. These end caps are molded in such a shape that a parallel beam of particles traverses the same thickness of plastic regardless of its distance from the center axis. The counter is mounted off-center relative to the end caps so that charged-particle-equilibrium is enhanced in one direction, and wall effects can be studied by simply turning the counter 180° . The physical size was chosen with a view to making measurements adequate for most purposes, with a reasonably portable, self-contained instrument. Some initial comparisons are reported in section PM-2 of this report.

Cynthia Counter

This design evolved from the realization that the lineal energy spectra of photons having energies of 20-100 keV may be seriously distorted by the presence of the stainless steel wires ordinarily used as one or more of the electrode structures with a counter. Evidence for such distortion exists in the measurements of photon spectra taken by R. Dvorak and published recently (1).

Current biological work with ^{125}I discussed elsewhere in this report makes it desirable to do accurate microdosimetric measurements on this isotope, whose photon spectrum falls within the region of worst distortion.

This distortion results from the fact that the photoelectric cross-section (which varies as $\sim z^4$) will be much higher for the steel structures than for the low - z tissue -equivalent plastic or gas. The higher energy photoelectron emitted by the steel wires will produce a slowing down spectrum in the vicinity of the

Figure 2: Heavy ion counter, cut-away view. Tissue-equivalent parts solid black.

collecting volume which is, on the average, too energetic.

Thus, it is desirable to construct a counter which is entirely non-metallic, i.e., made entirely of low-z materials. The resulting design is shown in Fig. 3. The helix is a special design, in a geometry in which two concentric helices run in opposite directions. The "wave-length" of the helices differ in the clockwise and counter-clockwise direction so that regular periodic nodes do not appear. The pitches are 7/inch and 8/inch. The dimensions of the strands are 0.002 in. x 0.004 in. The spherical grid defining the outer boundary of the collecting volume is a design which has been developed over many years and used in several wall-less type spherical counters. It consists of three sets of co-axial circular rings inscribed on the surface of a one-inch diameter sphere along mutually perpendicular axes. The struts produced by the injection molding process are 0.008 in. deep by 0.004 in. wide. Transparency of this grid is $\sim 92\%$.

The material of the helix and the spherical grid is C-552 air equivalent plastic. This was chosen because 1) at ~ 30 keV (^{125}I energy) the mass energy absorption coefficient of this plastic is actually closer to tissue than the standard A-150 tissue-equivalent plastic, and 2) it is structurally stronger than the A-150, especially important for the delicate helix. These properties more than compensate for a slightly more difficult molding technique required.

After many attempts and failures at making the center collecting electrode of various fibers, especially carbon, it was decided to try a fine human hair, made conducting by a graphite coating. The donor, a young lady named Cynthia, thus also gave the counter its appropriate name. The idea proved to be successful after proper techniques for coating the hair were developed. The hair diameter is 0.0035 inch.

The resulting counter is housed in a four-inch diameter spherical shell, $\frac{1}{4}$ in. thick, the same shell as used for previous wall-less counters of more conventional

Figure 3: "Cynthia" counter, cut-away view. Tissue-equivalent parts solid black.

design, but utilizing the same C-552 plastic as the grid structure.

Although no data have yet been taken with this counter, preliminary tests with aluminium x-rays and alpha sources show the counter to have exceptionally good resolution and gain for a counter of this type.

1/4 Inch S in S

The counter described here and shown in Fig. 4 is of conventional wall-less construction, usually dubbed "S in S", for "sphere within sphere". It is similar in design to the one-inch described by Kliauga and Dvorak (1). It is included here for completeness, and differs only in that the inner sphere is $\frac{1}{4}$ the size of the previously described counter, allowing four times the gas pressure for a given simulated tissue diameter, and thus, an equivalently larger distance to the outer wall. It has excellent resolution and gain, and was initially used at the Berkeley-Bevalac.

Neutron Counter

A recent design (Fig. 5) used at the Radiological Research Accelerator Facility by L. Goodman and D. Srdoc, this is essentially an "S in S" type of design utilizing the $\frac{1}{4}$ inch diameter wall-less counter as above, but housed in a two-inch aluminium shell. The dimensions were chosen with a view toward increasing neutron flux at low energies, by being able to move the counter in closer to the target.

The shell is fitted with interchangeable A-150 tissue-equivalent plastic liners of different thicknesses to allow for charged particle equilibrium studies.

1/16 Inch Cylindrical Counter

This counter (Fig. 6) is a cylindrical wall-less design housed in an A-150 plastic tube. The wall-to-counter dimension divided by equivalent site diameter yields a ratio similar to the $\frac{1}{4}$ -inch S in S. It was originally developed for the microdosimetry of pion beams where its small external diameter permits use in phantoms. It has also been used for photon microdosimetry in water phantoms,

Figure 4: "S in S" counter, $\frac{1}{4}$ inch, cut-away view. Tissue-equivalent parts solid black

Figure 5: Neutron counter.

Figure 6: 1/16 inch cylindrical counter, cut-away view. Tissue-equivalent parts solid black.

work described elsewhere in this report.

Its small physical size makes it attractive for work such as this, but it has some disadvantages. The construction of this counter is particularly difficult and time-consuming. It is a delicate and "fussy" counter to operate, and very unforgiving of any mistakes. Gain is limited by breakdown problems due to close tolerances of the electrodes, and resolution is fair.

One-Millimeter Counter

This counter represents still another step in the continuing movement toward miniaturization in counter construction. This counter, shown in Figs. 7a and 7b, has an active diameter of one millimeter diameter by one millimeter length. This volume is a cylindrical space in a solid tissue-equivalent plastic block. Passageways for flowing the counter gas through the volume and for an alpha-emitting calibration source are made in the block. In order to preserve field continuity, the region in which the cut for these passages intersects the cylindrical counting volume has had bars of the same tissue-equivalent material molded integrally into the opposing faces. The entire counter was constructed in a mold in which the collecting volume and spaces between these bars were etched out.

This counter is in the final phases of assembly and has not yet been tested. The extremely small sensitive volume is required for mixed-field microdosimetry, in the beam of electron accelerators where neutron doses are only of the order of 1% compared with low-LET doses. The fluence of protons should therefore be only of the order of 10^{-4} times that of the electrons. An additional feature is that the temporal distribution of the radiation is such that it occurs in bursts of the order of $1 \mu s$ at a frequency of 180 Hz . It is anticipated that measurements will be carried out at such dose rates that an average of some 10 electrons per pulse will traverse the counter and that the resultant distribution will differ substan-

Figure 7a: One millimeter counter, cut-away view. Tissue-equivalent parts solid black.

scale 100:1

Figure 7b: Detail of center section of one millimeter counter.

stantially in the rare case when a heavy particle appears as well. The lineal energy spectrum produced by the neutrons should be attainable by appropriate analysis.

We wish to acknowledge the skillful workmanship by Mr. Gary Johnson who is responsible for most of the actual construction involved in fabrication of these counters.

References

1. Kliauga, P. and Dvorek, R. Radiat. Res. 73:1 (1978)

Dusan Srdoc

The neutron microdosimetric measurements, reported in Ref. 1, included among other objectives a series of photon spectra obtained with an experimental setup identical to that of the neutron experiments. The primary reason for obtaining these spectra was related to the necessity of subtracting the gamma-ray component from the neutron microdosimetric distributions. As a natural extension of this work a number of independent microdosimetric spectra have been obtained for ^{60}Co and ^{137}Cs . These measurements constitute the subject of this report.

EXPERIMENTAL PROCEDURE

The experimental arrangement, the counter calibration procedure and data processing are similar to those described in Ref. 1. A ≈ 50 mCi ^{60}Co source and a ≈ 1 mCi ^{137}Cs source were used in a scatter-free environment to irradiate wall-less spherical proportional counters. Two counters, of similar design were used: the larger one was 1 inch in diameter and the smaller one was 1/4 inch in diameter. The spectra obtained at pressures corresponding to the same site size were almost identical for the two detectors. The "1 inch" counter was more convenient for simulating volumes larger than $2\text{ }\mu\text{m}$ in diameter in order to avoid high gas pressures, whereas the "1/4 inch" counter was used below $2\text{ }\mu\text{m}$. This latter counter was essential for the measurements in the region below 500 nm.

RESULTS

Microdosimetric spectra of ^{60}Co and ^{137}Cs gamma rays for simulated spherical volumes ranging from 60 nm to $8\text{ }\mu\text{m}$ in diameter were measured. The results are presented in the form of $y_d(y)$ vs $\log y$ plots and the mean values

$\bar{y}_F$ and $\bar{y}_D$ are tabulated. Full lines on the graphs represent the experimental data; broken or dotted lines are extrapolations. Calculations of the mean values $\bar{y}_F$ and $\bar{y}_D$ were done using the extrapolated spectra. In the tables, the values in parentheses indicate larger uncertainties due to extrapolated section at the low lineal energies for spectra below 250 nm.

The $y_d(y)$ vs $\log y$ spectra of ^{60}Co gamma rays and site sizes ranging from 125 nm to 1 μm are shown in Fig. 1. For the 60 nm spectrum only the high lineal energy region is shown in Fig. 1; the low and medium lineal energy regions are below the electronic noise. An attempt to increase the gas gain at this extremely low gas pressure for a proportional counter ($p_{60\text{ nm}} = 4.1$ Torr in the "1/4 inch" counter) resulted in frequent breakdowns. However, the equal spacing observed at the high lineal energy edge of spectra corresponding to site sizes differing by a factor of 2, seems to indicate that the spectra have the same general shape over the whole region, from 1 μm down to 60 nm. There is also a consistent trend in the $\bar{y}_D$ vs site size plot as shown in Fig. 3. Fig. 2 shows the $y_d(y)$ vs y spectra of ^{60}Co and site sizes above 0.5 μm as the parameter.

Several measurements (2, 3, 4, 5) of ^{60}Co gamma ray microdosimetry spectra are compared with those of M.H. Biavati et al. (2). The mean values $\bar{y}_F$ and $\bar{y}_D$ from Table 1 are plotted versus the site size in Fig. 3. A fairly good agreement can be seen, especially if the data are separated into two groups, obtained by using the walled or the wall-less counter. The mean values $\bar{y}_F$ and $\bar{y}_D$ for ^{137}Cs gamma rays and a range of site diameters are compared in Table 2 with several recent measurements (4, 5, 7). Again, the agreement is better if the walled and wall-less data are compared separately. The $y_d(y)$ vs $\log y$ spectra for ^{137}Cs are similar to those for ^{60}Co . Fig. 4

Figure 1: ^{60}Co gamma rays $y_d(y)$ vs $\log y$ spectra. Spherical site diameters: $1\mu\text{m}$, 500 , 250 , 125 , and 60 nm .

Figure 2: ^{60}Co gamma rays, $y_d(y)$ vs $\log y$ spectra. Spherical site diameters: 1 , 2 , 4 , and $8\mu\text{m}$.

Table 1
Comparison of experimental data
⁶⁰Co gamma rays, $\bar{y}_F$ and $\bar{y}_D$ values for various
site diameters

Site size (μ)	M.H.Biavati(6) 1966 (walled counter)	Braby Ellet(3) (Wall- less)	Dicello(2) 1974 (Walled counter)	Kliauga Dvorak(4) 1978 (Wall- less)	Varma et al.(5) 1980 (Walled counter)	This Report (Wall- less)
0.125	$\bar{y}_F$ - $\bar{y}_D$ -	- -	- -	- -	- -	- (4.0)
0.25	$\bar{y}_F$ - $\bar{y}_D$ -	- -	- -	0.549 3.04	- -	0.71 3.13
0.5	$\bar{y}_F$ 0.744 $\bar{y}_D$ -	(.58 μ) 0.479 2.17	0.47 2.8	0.36 2.10	0.475 2.66	0.40 2.27
1.0	$\bar{y}_F$ 0.393 $\bar{y}_D$ 1.86	(.92 μ) 0.38 1.66	- -	0.28 1.59	0.39 1.81	0.30 1.70
2.0	$\bar{y}_F$ 0.345 $\bar{y}_D$ 1.47	(1.84 μ) 0.36 1.51	0.23 1.3	0.255 1.22	0.368 1.50	0.30 1.27
4.0	$\bar{y}_F$ 0.345 $\bar{y}_D$ 1.14	- -	- -	0.256 0.974	- -	0.25 0.94
8.0	(7 μ) $\bar{y}_F$ 0.344 $\bar{y}_D$ 0.935	- -	- -	0.256 0.783	- -	0.23 0.69

Figure 3: Log of mean values y_F and y_D vs log of spherical site diameter in μm .

Figure 4: ^{137}Cs gamma rays, $yd(y)$ vs log y spectra. Spherical site diameters: 1, 2, 4, and 8 μm .

Table 2

Comparison of experimental data
 ^{137}Cs gamma rays, $\bar{y}_F$ and $\bar{y}_D$ values for
various site diameters

Site size (μ)		Kliauga Dvorak (4) (Wall-less)	Saq'an Haque (7) 1979	Varma et al.(5) 1980 (Walled)	This Report (Wall-less)	
0.25	y_F	0.712	-	-	0.73	
	$\bar{y}_D$	3.47	-	-	3.53	
0.5			Wall	W-less		
	$\bar{y}_F$	0.452	0.42	0.42	0.547	0.46
	$\bar{y}_D$	2.45	2.18	2.63	3.03	2.36
1.0	$\bar{y}_F$	0.371		0.494	0.43	
	$\bar{y}_D$	1.85		2.25	1.88	
2.0	$\bar{y}_F$	0.355	0.36	0.31	0.474	0.39
	$\bar{y}_D$	1.47	1.41	1.32	1.87	1.45
4.0	$\bar{y}_F$	0.350				0.34
	$\bar{y}_D$	1.19				1.11
8.0	$\bar{y}_F$	0.353				0.33
	$\bar{y}_D$	0.992				0.89

shows $y_d(y)$ spectra for site diameters ranging from 1 to 8 μm , whereas Fig. 5 shows the same for 1, 0.5 and 0.250 μm site diameters. The ^{60}Co and ^{137}Cs spectra for 1 μm site are compared in Fig. 6. As expected, the ^{137}Cs spectrum for the same site size is shifted slightly to the right, i.e., toward the higher lineal energy, because of higher specific ionization of less energetic gamma rays.

REFERENCES

1. D. Srdoc, L.J. Goodman, S.A. Marino and M. Zaider: Neutron microdosimetry at RARAF, this Report.
2. J.F. Dicello: Event distributions for ^{60}Co gamma rays, Annual Report on Research Project, COO-3243-3, Columbia Univ., 78-87 (1974).
3. L.A. Braby and W.H. Ellett: Ionization in microscopic volumes irradiated by energetic photons, Oregon State Univ., Report AD 731709 (1971).
4. P. Kliauga and R. Dvorak: Microdosimetric measurements of ionization by monoenergetic photons, Radiat. Res., 73, 1-20 (1978).
5. M.N. Varma, J.W. Baum and P. Kliauga: Microdosimetric measurements for photons in a water phantom, To be presented at the 7th Symp. on Microdosimetry, Oxford/United Kingdom, Sept. 8-12, 1980.
6. M.H. Biavati and E. Boer: $D(y)$ -spectra of gamma-rays, Annual Report on Research Project, NYO-2740-3 Columbia Univ., 87-101, (1966).
7. S.A. Saq an and A.K.M. Haque: Microdosimetric studies with low level LET radiation using cylindrical walled and wall-less proportional counters, Nucl. Instru. Meth., 167, 431-437, (1979).

Figure 5: ^{137}Cs gamma rays, $yd(y)$ vs $\log y$ spectra. Spherical site diameters: 0.25, 0.5 and 1μ .

Figure 6: 1μ site diameter; comparison of ^{60}Co and ^{137}Cs gamma ray $yd(y)$ vs $\log y$ spectra.

BIOPHYSICS

- BP-1 Statistical analysis of cell survival data: A binary approach
Marco Zaider
- BP-2 Algorithms for determining the proximity distribution from
variance measurements
Marco Zaider (in collaboration with K. Hanson and G.N. Minerbo,
Los Alamos Scientific Laboratory)
- BP-3 Proximity function for low energy electrons in nitrogen - applica-
tion to Iodine-125
Marco Zaider
- BP-4 Saturation effects for sparsely ionizing particles
Marco Zaider and Harald H. Rossi
- BP-5 Sequential exposures to mixed fields of radiation: A theoretical
approach
Marco Zaider and Harald H. Rossi
- BP-6 Computer Activities
Marco Zaider and Paul J. Kliauga

Introduction

The statistical analysis of cell survival curves has been the object of several studies in the past years (1,2,3). Two main approaches have been proposed:

- a) the non-parametric method in which one simply fits a set of experimental data points subject to only very general restrictions such as monotony and convexity (from above). No analytical expression for the dose-effect relation is assumed.
- b) regression or analysis of variance techniques: in this approach the method of least squares (MLS) is applied to an appropriate linear model and relevant parameters are determined. In this report the applicability of the MLS to fitting linear-quadratic dose-effect models is examined. A third approach, based on the maximum likelihood method (MLM) and valid under more general conditions is discussed and a comparison is made between the MLS and the MLM based on the analysis of a set of experimental survival data.

Brief Theoretical Review

a) Regression analysis

Consider a set of n independent random variables, $y_1, y_2, \dots, y_n$ having variances all equal to σ^2 :

$$\text{cov}(y_i, y_j) = 0 \quad \text{var}(y_i) = \sigma^2 \quad i \neq j = 1, \dots, n \quad (1)$$

and means given by the regression function:

$$E(y_i) = \beta_1 x_{1i} + \beta_2 x_{2i} + \dots + \beta_k x_{ki} \quad (k < n) \quad (2)$$

(x_{ij}) are known constants and $\beta_1, \dots, \beta_k$ are unknown parameters to be estimated (also called regression coefficients).

Under the conditions of Eqs. (1) and (2) the following result, known as the

Gauss-Markov theorem holds (See Ref. 4):

"The minimum variance linear estimators of the regression coefficients β_i are b_i ($i = 1, \dots, k$) where:

$$b_i = \sum_j a^{ij} a_{jo} \quad (3)$$

and

$$a_{jo} = \sum_i x_{ji} y_i \quad (4)$$

$$a_{ij} = \sum_m x_{im} x_{jm} \quad (5)$$

$$||a^{ij}|| = ||a_{ij}||^{-1} \quad (6)$$

The covariance matrix of the b_i is $||a^{ij} \sigma^2||$.

It can also be shown that b_i as given by Eq. (3) are identically the same with the values of β_i which minimize the sum of squares:

$$Q(\beta_i) = \sum_i (y_i - \beta_1 x_{1i} - \dots - \beta_k x_{ki})^2 \quad (7)$$

This last result is commonly known as the MLS. Under the further assumption that the random variables y_i are normally distributed with probability distributions:

$$N(\beta_1 x_{1i} + \dots + \beta_k x_{ki}, \sigma^2) \quad (8)$$

confidence intervals for β_i can be calculated.

Indeed

$$b_i \pm (t_{n-k, \gamma}) \frac{\sqrt{a^{ii} Q(b_i)}}{n - k} \quad (9)$$

is a 100% γ confidence interval for β_i , where $t_{n-k, \gamma}$ satisfies:

$$\int_{-t_{n-k, \gamma}}^{t_{n-k, \gamma}} f_{n-k}(t) dt = \gamma \quad (10)$$

and f_{n-k} is the probability distribution function of the "Student" distribution with $(n-k)$ degrees of freedom. Finally, a joint 100% γ confidence region (ellipsoid) for the parameter point $(\beta_1, \dots, \beta_k)$ has the equation:

$$\sum_{ij} a_{ij} (\beta_i - b_i) (\beta_j - b_j) = \frac{1}{n-k} kQ(b_i) F_{k,n-k,\gamma} \quad (11)$$

where $F_{k,n-k,\gamma}$ is calculated from the Snedecor distribution:

$$\int_0^{F_{k,n-k,\gamma}} dF_{k,n-k}(f) = \gamma \quad (12)$$

One should note the importance of the constant variance condition Eq.(1) for estimating the regression coefficients and the normality requirement Eq.(8) for calculating confidence intervals and joint confidence regions.

b) The maximum likelihood method

Suppose $y_1, \dots, y_n$ are a set of independent random variables with parent distributions $f_i(y_i, \beta_1, \dots, \beta_k)$, $i = 1, \dots, n$ where β_i are unknown parameters. Given a sample $y_1, \dots, y_n$, the likelihood function, L , is defined as:

$$L(\beta_1, \dots, \beta_k) = \prod_{i=1}^n f_i(y_i, \beta_1, \dots, \beta_k) \quad (13)$$

The MLM consists of making the assumption that the observed set of measurements is more likely to have come from the actual parent distributions such that if $\beta_1, \beta_2, \dots, \beta_k$ are the real coefficients, for any other set of coefficients, β'_i , one has

$$L(\beta_1, \dots, \beta_k) \geq L(\beta'_1, \dots, \beta'_k) \quad (14)$$

The MLM amounts to finding those values of b_i which maximize L , or solving the system of equations:

$$\frac{\partial \log L(\beta_1, \dots, \beta_k)}{\partial \beta_i} \Big|_{\beta_i = b_i} = 0 \quad (15)$$

since maximizing a function or its logarithm leads to equivalent results. (The reason for using the logarithm, besides the simplification of transforming a product into a summation, is related to applying the central limit theorem for estimating the variance of the parameters.)

Asymptotically, the estimators $(b_1, \dots, b_k)$ are normally distributed with mean $(\beta_1, \dots, \beta_k)$ and covariance matrix $||v_{ij}||^{-1}$, where:

$$v_{ij} = -E \left(\frac{\partial \log L}{\partial \beta_i \partial \beta_j} \right) \quad (16)$$

a joint, approximate 100% γ confidence region for β_i is given by:

$$\sum v_{ij} (\beta_i - b_i) (\beta_j - b_j) = \chi^2 (\gamma; k) \quad (17)$$

where $\chi^2 (\gamma; k)$ is the γ percentage point of the chi-square distribution with k degrees of freedom. It should be mentioned that if the random variables y_i are normally distributed, the MLM and the MLS are equivalent.

In most cases, the system of equations (15) does not provide closed form expressions for the estimators b_i . Numerical techniques are available for solving these equations. One of the most common numerical methods, the Newton-Raphson procedure, will be presented in the following.

Let $g_i(b_1, \dots, b_k)$ be defined as:

$$g_i(b_1, \dots, b_k) = \frac{\partial \log L (\beta_1, \dots, \beta_k)}{\partial \beta_i} \Big|_{\beta_i = b_i} \quad (18)$$

Suppose $b_1^{(0)}, \dots, b_k^{(0)}$ is the set of initial estimates of $\beta_1, \dots, \beta_k$. Then the n th iteration $b_1^{(n)}, \dots, b_k^{(n)}$ to the solution is:

$$b_j^{(n)} = b_j^{(n-1)} + \sum_{i=1}^k g_i^{(n-1)} ||v_{ij}||^{-1}_{(n-1)} \quad (19)$$

$j = 1, \dots, k$

The iteration procedure is stopped when $\log L$ does not increase appreciably or when

the difference $(b_j^{(n)} - b_j^{(n-1)})$ is smaller than a given limit. The Newton-Raphson procedure is very sensitive to the choice of the initial estimates, $b_i^{(0)}$. Normally one uses the least squares estimates as a starting point, although the use of more than one initial set is recommended.

Binary Analysis of Survival Data

In a typical cell-survival experiment a vial containing a large number of cells (about 10^6) is exposed to a measured amount of radiation. Subsequently, by dilution, a relatively small number of cells (200-1000) are drawn off, plated out onto a Petri dish, provided with growth medium, and given time to reproduce. By dividing five or more times, the cells form colonies which are counted and the number compared with a control sample of unirradiated cells.

Consider a control experiment where N cells are diluted and plated and let n be the number of colonies obtained. This number is a random variable which has a binomial distribution.

$$P_B(n|N) = \binom{N}{n} p_1^n (1 - p_1)^{N - n} \quad (20)$$

where p_1 is the probability of obtaining a colony per initial cell. The average number of colonies is Np_1 . Under normal experimental conditions, N is very large and p_1 small, such that $P(n)$ can be treated as a Poisson distribution.

$$P_p(n|\bar{n}) = \frac{\bar{n}^n e^{-\bar{n}}}{n!} \quad (21)$$

with

$$\bar{n} = p_1 N \quad (23)$$

If the same sample of N cells is now irradiated, and if p is the probability of survival per cell after a dose D , one has for the probability of x surviving cells:

$$P(x) = \sum_{n=x}^{\infty} P_p(n|\bar{n}) P_B(x|n) \quad (24)$$

where

$P_p(n|\bar{n})$ is the probability of n control cells when the average control number is $\bar{n}$

$P_B(x|n)$ is the probability of x surviving cells when n cells are irradiated.

In a more explicit form:

$$P(x) = \sum_{n=x}^{\infty} \frac{\bar{n}^n e^{-\bar{n}}}{n!} \binom{n}{x} p^x (1-p)^{n-x} \quad (25)$$

By simple manipulations it can be shown that:

$$P(x) = \frac{e^{-\bar{pn}} (\bar{pn})^x}{x!} \quad (26)$$

which is in fact a Poisson distribution with average $\bar{pn}$. If the number of control cells is fixed (not random), then $P(x)$ becomes:

$$P_n(x) = \binom{n}{x} p^x (1-p)^{n-x} \quad (27)$$

In the limiting case where the number of cells available becomes very large, and the probability of survival is large enough so that $np \gg 1$, the distribution (27) can be approximated by a Gaussian distribution. For the rest of the report, the assumption will be made that p has the form resulting from the theory of dual radiation action, namely:

$$p = e^{-\alpha D - \beta D^2} \quad (28)$$

where α and β are parameters to be estimated.

Regression Analysis of Survival Data - A Critical View

Consider a set of measured survival fractions, y_i , corresponding to the dose levels, D_i . The problem consists of estimating the parameters α, β of Eq.(28) such that

$$Q = \sum (y_i - e^{-\alpha D_i - \beta D_i^2})^2 \quad (29)$$

is minimized. In order to apply the regression technique, one has to "linearize" the equation (28) in terms of α, β and therefore one has to consider

$$Q' = \sum (-\log y_i + \alpha D_i + \beta D_i^2)^2 \quad (30)$$

Essentially, a new random variable, $z_i = \log y_i$ is introduced which has quite different distribution compared to the distributions originally attributed to y Eqs.(26) or (27). In fact, the application of regression analysis to the variables (z_i) has the following important limitations:

a) The condition Eq.(1) of constant variance required by the Gauss-Markov theorem

cannot hold except for the very peculiar case where $(\bar{n}_i p_i)$ are all about the same (for the Poisson distribution), or when p_i are all in the range where $p_i(1-p_i)$ changes relatively little (for the binomial distribution). This range is roughly

$$0.2 \leq p_i \leq 0.8$$

b) Because z_i are not normally distributed, neither one of the methods available to estimate confidence intervals for the parameters α and β are applicable. In spite of these restrictions, the MLS has been frequently applied, most of the time as a curve fitting method. As mentioned previously, the results of such a procedure may be used as initial values for iteration techniques based on the MLM approach. Where precise estimation of the parameters α and β is required, a more rigorous analysis method should be used.

Maximum Likelihood Analysis of Survival Data

The experiment under consideration consists of irradiating groups of $n_1, \dots, n_k$ cells to the dose levels, $D_1, \dots, D_k$ (in fact, as mentioned previously, n_i are random variables resulting from the process of dilution and plating). After irradiation, $x_1, x_2, \dots, x_k$ surviving colonies are observed, respectively. The maximum likelihood function is

$$L = \prod_{i=1}^k \frac{e^{-p_i n_i} (p_i n_i)^{x_i}}{x_i!} \quad (31)$$

with

$$p_i = e^{-\alpha D_i} - \beta D_i^2 \quad (32)$$

α and β can be found by solving the system of equations:

$$\begin{aligned} \frac{\partial \log L}{\partial \alpha} &= \sum \left[-n_i \frac{\partial p_i}{\partial \alpha} + \frac{x_i}{p_i} \frac{\partial p_i}{\partial \alpha} \right] = 0 \\ \frac{\partial \log L}{\partial \beta} &= \sum \left[-n_i \frac{\partial p_i}{\partial \beta} + \frac{x_i}{p_i} \frac{\partial p_i}{\partial \beta} \right] = 0 \end{aligned} \quad (33)$$

Using (37) one obtains:

$$\begin{aligned}\sum [n_i D_i p_i - x_i D_i] &= 0 \\ \sum [n_i D_i^2 p_i - x_i D_i^2] &= 0\end{aligned}\tag{34}$$

Following the procedures described in Sec II b., one obtains the following iteration equations:

$$\begin{aligned}\alpha_i &= \alpha_{i-1} + \frac{1}{||v||} (v_{00} v_{22} + b_1 v_{22} + v_{11} v_{12} - b_2 v_{12}) \\ \beta_i &= \beta_{i-1} + \frac{1}{||v||} (-v_{00} v_{12} - b_1 v_{12} - v_{11}^2 + b_2 v_{11})\end{aligned}\tag{35}$$

where:

$$\begin{aligned}b_1 &= \sum x_i D_i & b_2 &= \sum x_i D_i^2 \\ v_{00} &= \sum p_i n_i D_i & ||v|| &= v_{11} v_{22} - v_{12}^2 \\ v_{11} &= \sum p_i n_i D_i^2 & v_{12} &= \sum p_i n_i D_i^3 & v_{22} &= \sum p_i n_i D_i^4\end{aligned}\tag{36}$$

The variances of α and β are

$$\sigma_\alpha^2 = \frac{v_{22}}{||v||} \quad \sigma_\beta^2 = \frac{v_{11}}{||v||}\tag{37}$$

and the joint 100% γ confidence ellipse is:

$$v_{11} (\alpha - \bar{\alpha})^2 + v_{22} (\beta - \bar{\beta})^2 + 2v_{12} (\alpha - \bar{\alpha}) (\beta - \bar{\beta}) = \chi^2(\gamma, 2)\tag{38}$$

A Comparison of the MLS and the MLM Results

A set of track segment survival curves (kindly provided by Dr. R.P. Bird) was analyzed using the MLS and the MLM techniques. The estimated values for α and β together with their 95% confidence errors are presented in the Table. Also shown are the results of a third calculation, using a non-linear least square estimation method. The calculated values for α and β using the three different methods are in good agreement (with only one exception). This reflects the correctness of using the MLS as a fitting procedure. On the other hand, the MLS seems to overestimate (by factors of 2 to 3) the confidence intervals for the parameters. Whether this is true in general or only in this particular case, is not clear at this point.

Table BP-1-1

1st row: least square fit
 2nd row: maximum likelihood
 3rd row: non-linear x^2 fit

RUN	LET (kev/ μ m)	$\alpha \times 10^3 \text{ rad}^{-1}$	$\beta \times 10^6 \text{ rad}^{-2}$
83-1	10	3.99 ± 0.69	2.72 ± 1.06
		4.04 ± 0.41	2.65 ± 0.62
		4.24 ± 0.45	2.37 ± 0.67
74-1	20	4.97 ± 0.88	5.23 ± 1.40
		4.97 ± 0.43	5.25 ± 0.70
		5.24 ± 0.45	4.87 ± 0.73
71-1	33	7.73 ± 1.45	4.61 ± 2.45
		6.83 ± 0.47	7.43 ± 0.89
73-1	40	8.54 ± 1.12	11.5 ± 3.5
		8.47 ± 0.60	11.8 ± 1.8
		8.55 ± 0.63	11.7 ± 1.9
89-1	68	11.65 ± 2.32	6.29 ± 8.16
		11.66 ± 0.79	6.38 ± 2.79
		11.43 ± 0.87	8.20 ± 3.14
81-1	90	15.37 ± 1.12	0.63 ± 3.46
		15.42 ± 0.45	0.81 ± 1.48
		16.17 ± 0.49	-1.01 ± 1.59
82-1	127	12.76 ± 1.93	4.39 ± 6.06
		12.63 ± 0.69	4.34 ± 2.17
		13.07 ± 0.35	3.99 ± 1.11
84-1	170	14.88 ± 2.13	-5.81 ± 7.13
		14.75 ± 0.71	-5.77 ± 2.73
		13.87 ± 0.13	-1.42 ± 0.43

The confidence intervals are unexpectedly small if one considers the large fluctuations in the experimental data. This may be a result of the relative lack of sensitivity of the calculated survival fractions to variations in the parameters.

Finally, Fig. 1 shows a dramatic (although untypical) example of the difference between the two approaches, as reflected in the 95% confidence ellipses for RUN 71-

1.

References

1. A.M. Kellerer and J. Brenot, Rad. and Environm. Biophys. 11: 1-13 (1974).
2. A.M. Kellerer and J. Brenot, Rad. Res. 56: 28-39 (1973).
3. J.W. McLarty and H.D. Thames, Jr., Rad. Res. 69: 1-15 (1977).
4. S.S. Wilks, Mathematical Statistics, John Wiley and Sons, Inc., NY-London, 1962.

Figure 1: 95% confidence regions for the parameters α and β calculated using the maximum likelihood method (MLM) and the method of least squares (MLS) as applied to the data of RUN 71-1.

BP-2 Algorithms for Determining the Proximity Distribution from Variance Measurements

M. Zaider in collaboration with K. Hanson and G.N. Minerbo (Los Alamos Scientific Laboratory)

Introduction

In recent developments in theoretical microdosimetry (1) the concept of proximity distribution has been introduced. This concept refers to two different aspects in the process of cellular lesion-induction by ionizing radiation: the geometrical distribution of sensitive loci in the cell (called $s(x)$) and the inchoate pattern of elementary energy transfers, characterized by the function $t(x)$.

The function $s(x)$ characterizes the sensitive structure of the biological object. It is, therefore, independent of the irradiating field and will not be considered in the present article.

In Ref. 1 the following definition of $t(x)$ is given:

"Select energy transfers, e , randomly in the irradiated medium. Then $t(x) dx$ is the expected sum of energy transfers contained in a shell of radius x and thickness dx centered at the transfer point where the energy transfer e occurred."

As mentioned in the same article (1) transfer points are selected with a probability proportional to the energy transferred, e . The biophysical significance of the proximity distribution, $t(x)$, is apparent in the context of the generalized formulation of the dual radiation action (1).

Indeed, it was shown that the mean number of lesions per cell can be calculated from the expression:

$$\bar{\epsilon}(D) = k (\xi D + D^2) \quad (1)$$

where the coefficient of the linear term, ξ , depends directly on the proximity function, $t(x)$. Calculating, or measuring, the distribution $t(x)$ becomes, therefore, a primary goal for microdosimetry-based biophysical considerations.

To date no experimental methods are available for directly measuring $t(x)$. As one alternative, a number of calculations, utilizing the Monte Carlo technique, have been performed (2-4). The main restrictions associated with the theoretical approach are related to the limited present understanding of the basic physical interactions at the molecular level. In addition, Monte Carlo techniques are frequently hindered by the exceedingly large amounts of computer memory space and running times necessary. Finally, direct comparisons between theoretical results and experiment are rather restricted, for obvious reasons.

In the present paper an algorithm is proposed which allows the calculation of the distribution $t(x)$ from measurements based on an already available experimental technique, namely the variance method of Bengtsson and Lindborg (5). This algorithm is based on a very important theoretical relation, obtained by Kellerer and Chmelevski (6) and which relates the proximity function to the dose average specific energy, $\bar{z}_D$:

$$\bar{z}_D = \frac{1}{m} \int_0^r \left[1 - \frac{3x}{2r} + \frac{x^3}{2r^3} \right] t(x) dx \quad (2)$$

Where the quantity in parentheses is related to the distribution of distances between two random points in a sphere of diameter r .

In the next section the Eq. (2) will be demonstrated again in a rather elementary fashion in order to alleviate for the reader the difficulty of going over the rather sophisticated developments of Kellerer and Chmelevski. The variance method is shortly reviewed next. Finally, the proposed algorithm is discussed in the last section.

The Formula for $\bar{z}_D$

Consider a spherical volume of diameter r . Let the center of this sphere be x_i . This sphere may represent the active volume of a proportional detector (the assumption of a spherical shape is not necessary for the demonstration; it will be assumed though for simplicity). If a particle traverses this volume one has the following expression for the specific energy:

$$z(x_i) = \frac{1}{m} \sum_{k=1}^{n_i} e_k \quad (3)$$

Here e_k are energy transfers inside the sphere. If now this experiment is repeated n times (or if one considers n spheres, each traversed by one particle) one can calculate the dose average specific energy, $\bar{z}_D$:

$$\bar{z}_D = \frac{\sum_{i=1}^n z^2(x_i)}{\sum_{i=1}^n z(x_i)} \quad (4)$$

or:

$$\bar{z}_D = \frac{\sum_{i=1}^n \left[\sum_{j,k=1}^{n_i} e_j e_k \right]}{\sum_{i=1}^n \left[\sum_{k=1}^{n_i} e_k \right]} \quad (5)$$

In a Monte Carlo scheme the summation over "i" is equivalent to placing randomly spheres of diameter r in the associate volume of a track (i.e., with their centers in the associate volume; for a definition of the associate volume see Ref. 7). Then for each position of the sphere one calculates the sum over all cross products and individual energy transfers in Eq. (5).

Consider first the summation over e_k . Each transfer points, e_k may appear more than once in the sum over "i". Actually, *in average*, the number of times each point will appear will be equal to nV_r/V , where V_r is the volume of the sphere, V is the total associated volume, and n is the number of spheres considered. One has:

$$\frac{n}{\sum_{i=1}^n} \left[\sum_{k=1}^{n_i} e_k \right] = n \frac{V_r}{V} \sum_{k=1}^N e_k \quad (6)$$

with N , the total number of transfer points.

Consider now the summation over cross products. Each term, $e_j e_k$, will appear any time the sampling sphere has its center in the intersecting volume of two spheres centered at e_j and e_k . This volume is:

$$V_1 = V_r \left[1 - \frac{3x}{2r} + \frac{x^3}{2r^3} \right] \quad x = |x_j - x_k| \quad x \leq r$$

then, similar to Eq. (6):

$$\frac{n}{\sum_{i=1}^n} \left[\sum_{kj=1}^{n_i} e_j e_k \right] = n \frac{V_r}{V} \sum_{\substack{j,k \\ x \leq r}} e_j e_k \left[1 - \frac{3x}{2r} + \frac{x^3}{2r^3} \right] \quad (7)$$

Therefore:

$$\bar{z}_D = \frac{1}{m} \sum_{j,k} e_j e_k \left[1 - \frac{3x}{2r} + \frac{x^3}{2r^3} \right] / \sum e_k \quad x \leq r$$

If now k and j are restricted to points separated by distances between x and $x + dx$ one can write:

$$\bar{z}_D = \frac{1}{m} \int_0^r dx u(x) \left[\frac{1}{\sum e_k} \sum_{(k,j) \in [x, x+dx]} e_j e_k \right] \quad (8)$$

with $u(x) = V_1/V_r$.

The quantity in square brackets is the proximity function, $t(x)$, defined in the previous section. Indeed, with the notation

$$E = \sum e_k$$

one has:

$$t(x) = \sum_k \frac{e_k}{E} \left(\sum_j e_j \right) \quad (k,j) \in [x, x + dx] \quad (9)$$

which is the mathematical expression of the definition of $t(x)$. With these notations:

$$\bar{z}_D = \frac{1}{m} \int_0^r u(x) t(x) dx \quad (10)$$

equivalent to Eq. (2).

The Variance Method

The variance method of Bengtsson and Lindborg (5) allows the experimentalist to measure directly the dose average specific energy, $\bar{z}_D$, without the usual intermediate step of determining a complete microdosimetric distribution. This method is based on a simple expression relating, for a given absorbed dose, D , the relative variance (σ_r^2) of the specific energy distribution, $f(z;D)$ to $\bar{z}_D$:

$$\sigma_r^2 = \bar{z}_D / D \quad (11)$$

In principle one records specific energy spectra, for a fixed average dose, and the relative variance of this spectrum provides a direct measure of $\bar{z}_D$. Detailed experimental procedures have been described previously (5). The significant advantages of this method become apparent when one simulates very small object sizes: while the single event signals may be too small to be measured, the specific energy, z , resulting from numerous individual events will still be well within the range of detection. As an example, in a recent publication (8), values of $\bar{z}_D$ for Co^{60} and x-rays have been reported for site sizes as small as 10 nm in diameter (unit density material). The curves fitted to the experimental points, and reported in Ref. 8 are:

$$\text{X-rays: } \bar{e}_D = 2.79 r^{0.64} \quad (12)$$

$$\text{Co}^{60} : \bar{e}_D = 1.34 e^{0.60} \quad 0.01 < r < 22 \mu\text{m} \quad (13)$$

with $\bar{e}_D = \bar{z}_D \cdot m$, and $\bar{e}_D$ and r in units of keV and μm respectively. Analytical expressions such as those of Eqs. (12) and (13) could be used directly in the unfolding algorithm presented in the next section.

Algorithms for the Proximity Function

The problem addressed here is to find the proximity function, $t(x)$, from Eq. (2) when a set of measured values for $\bar{z}_D(r)$, or an analytical expression are available. The latter case will be treated first.

Assume the form of $\bar{e}_D(r)$ is known ($\bar{e}_D$ is the dose average energy deposited in the detector, i.e., $\bar{e}_D = \bar{z}_D \cdot m$). It can be easily verified that the solution of Eq. (2) is:

$$t(r) = \frac{1}{3} \frac{d^2}{dr^2} \left(\frac{1}{r^2} \left[\frac{d}{dr} r^4 \bar{e}_D(r) \right] - r \bar{e}_D(r) \right) \quad (14)$$

In particular, if $\bar{e}_D(r) = \alpha r^\beta$ one has:

$$t(r) = \frac{\alpha}{3} \beta (\beta + 1) (\beta + 3) r^{\beta-1} \quad (15)$$

A second solution can be given in terms of the Mellin transforms. This is based on the fact that the function $u(x,r)$ of Eq. (10) depends on x/r only.

Let us write:

$$e(r) = \int_0^\infty u\left(\frac{x}{r}\right) t(x) dx \quad (16)$$

with

$$u(x/r) = \begin{cases} u(x,r) & x \leq r \\ 0 & x > r \end{cases} \quad (17)$$

Let $E(\sigma)$, $U(\sigma)$ and $T(\sigma)$ be the Mellin transforms of $e(r)$, $u(r)$, and $t(r)$, respectively. For instance, by definition:

$$T(\sigma) = \int_0^{\infty} x^{\sigma-1} t(x) dx \quad (18)$$

$$t(x) = \frac{1}{2\pi i} \int_{c-i\infty}^{c+i\infty} x^{-\sigma} T(\sigma) d\sigma \quad (19)$$

Then, from Eq. (16) one has:

$$E(\sigma) = \int_0^{\infty} dr r^{\sigma-1} \int_0^{\infty} u\left(\frac{x}{r}\right) t(x) dx \quad (20)$$

or, by the change of variable $y = x/r$:

$$E(\sigma) = \int_0^{\infty} x^{\sigma} t(x) dx \int_0^{\infty} y^{-\sigma-1} u(y) dy \quad (21)$$

From Eq. (21), and using Eq. (18) one has:

$$E(\sigma) = T(\sigma+1) \cdot U(-\sigma)$$

or:

$$T(\sigma) = E(\sigma-1) / U(-\sigma+1) \quad (22)$$

The Eq. (22), in fact a well known result from the theory of Mellin transforms, shows that if one knows the transforms $E(\sigma)$ and $U(\sigma)$ one can calculate $T(\sigma)$ and the corresponding function $t(r)$.

In particular, if the expression of $u(x/r)$ for a sphere is used, one has:

$$U(-\sigma+1) = \int_0^1 y^{-\sigma} u(y) dy = \int_0^1 y^{-\sigma} \left[1 - \frac{3}{2}y + \frac{1}{2}y^3\right] dy \quad (23)$$

The Eq. (23) can be readily integrated and replacing the result in Eq. (22)

one obtains:

$$T(\sigma) = \frac{1}{3} (1-\sigma) (2-\sigma) (4-\sigma) E(\sigma-1) \quad (24)$$

or, using Eq. (13):

$$t(x) = \frac{1}{6\pi i} \int_{c-i\infty}^{c+i\infty} x^{-\sigma} (1-\sigma) (2-\sigma) (4-\sigma) E(\sigma-1) d\sigma \quad (25)$$

In the solution of Eq. (25) $t(x)$ is expressed as an integral over the Mellin transform of $\bar{e}_D(r)$. The advantages of this solution consists of: a) the possibility of using the experimental points, rather than an analytical fit, when calculating the integrals, and b) the strong relation, via Laplace transforms to existing integration algorithms.

It can be easily verified that if, again, one has $\bar{e}_D(r) = \alpha r^\beta$ one obtains from the Eq. (25) the solution (15).

Conclusion

Algorithms for the unfolding of proximity functions from measured values of $\bar{z}_D$ have been proposed. These algorithms should stimulate further use of the variance method. In addition, the proposed expressions should provide a further link between theoretical calculation of the proximity function and experiment.

From biophysical standpoint, the significance of the present results rests in the understanding of the complete equivalence between the dose average specific energies and the proximity distributions. This result has been postulated by Kellerer (9).

References

1. Kellerer, A.M. and Rossi, H.H. A generalized formulation of Dual Radiation Action. Radiat. Res. 75:471-488 (1978).
2. Paretzke, H.G. An appraisal of the relative importance for microbiology

- of the effects of slow electrons. In Proceedings, Fifth Symposium on Microdosimetry, Verbania - Pallanzo, Italy (J. Booz, H.G. Ebert, and B.G.R. Smith Eds.) pp. 41-58. Commission of the European Communities, Luxembourg, 1976.
3. Terrissol, M., Fourmenty, J. and Patau, J.P. Determination theorique des fonctions microdosimetriques pour des electrons de basse energie dans les gaz. In Proceedings, Fifth Symposium on Microdosimetry, Verbania-Pallanzo Italy (J. Booz, H.G. Ebert, and B.G.R. Smith, Eds.), pp. 393-406. Commission of the European Communities, Luxembourg, 1976.
 4. Turner, J.E., Hamm, R.N., Wright, H.A., Ritchie, R.H. and Katz, R. Calculated electron slowing-down spectra for liquide water irradiated by X and gamma rays. In Proceedings, Sixth Symposium on Microdosimetry, Brussels, Belgium (J. Booz and H.G. Ebert, Eds.) pp. 375-383, Harwood, London, 1978
 5. Bengtsson, L.G. and Lindborg, L. Comparison of pulse height analysis and variance measurements for the determination of dose mean specific energy. Proc. 4th Symposium on Microdosimetry, p. 823, EUR 5122 d-e-f (1974).
 6. Kellerer, A.M. and Chmelevski, D. Concepts of microdosimetry, III. Mean values of microdosimetric distributions. Rad. and Environm. Biophys. 12:321-335 (1975).
 7. Lea, D.E. Actions of radiation on living cells. Cambridge: Cambridge University Press (1946).
 8. Forsberg, B., Jensen, M., Lindborg, L. and Samuelson, G. Determination

of the dose mean specific energy for conventional x-rays by variance measurements. Proceedings of Sixth Symposium on Microdosimetry, Brussels (Belgium), EUR 6064 DE-EN-FR (1978).

9. Kellerer, A.M. Private communication.

BP-3 Proximity Function for Low Energy Electrons in Nitrogen -
Application to Iodine-125

M. Zaider

Introduction

The measurement and calculation of microdosimetric distributions and quantities at nanometer level constitute one of the major objectives of the biophysical research in this laboratory. From theoretical viewpoint, and in line with the latest developments in the Theory of Dual Radiation Action, part of the present effort is concentrated on calculating the so-called proximity functions of energy transfers, $t(x)$, for monoenergetic electrons.

There are several equivalent definitions for the distribution $t(x)$ (See Ref 1). In the present report the working (or operational) definition of Kellerer and Chmelevsky (1) will be used: this definition is related to the concrete calculation of the proximity functions via Monte Carlo techniques. Consider for this a track characterized by its transfer points and the associated energy transfers. Under the conditions of a uniform and isotropic irradiation field, the differential distribution, $t(x)$ can be calculated from:

$$t(x) = \sum e_i e_j / \sum e_k \quad (1)$$

where the sum over the energy transfers, e_k , extends to the whole track, and the sum over the cross products, $e_i e_j$, is limited to those transfer points whose distance (i,j) is between x and $x + dx$. In a normal calculation $t(x)$ is averaged over a number of particle tracks.

If the radiation field is characterized by a distribution of initial energies of the particles, one can obtain the proximity function from the expression:

$$t(x) = \int t_E(x) E_p(E) dE / \int E_p(E) dE \quad (2)$$

where $p(E)dE$ is the fraction of particles with energies between E and $E + dE$ and $t_E(x)$ is the proximity distribution for a particle of energy E .

It is obvious that, at least in principle, if distributions $t(x)$ are known for any electron energy, one should be able to predict the proximity functions for any mixed fields, including heavy particles (with the additional knowledge of the primary electron spectrum). From a practical viewpoint the calculation can be divided in four steps:

- a) calculate the initial energy spectrum $p(E)$
- b) simulate electron tracks for all the energies in the distribution $p(E)$
(i.e., calculate all the transfer points and energy transfers associated with a given track)
- c) calculate proximity functions, $t_E(x)$, from Eq.(1)
- d) calculate the proximity distribution $t(x)$, from Eq.(2)

In the present set-up a computer program is associated with each of the previous steps. Short descriptions of the codes follow in the next sections. As an application, the proximity function in a medium irradiated externally with I^{125} is calculated. This particular example is important in the context of the present radiobiological research with I^{125} in our laboratory.

The Initial Energy Spectrum of Electrons

The computer code PHOEL-2 (2), kindly provided by the Radiation Shielding Information Center, was used to calculate the energies of photoelectrons and Compton electrons in water irradiated by I^{125} photons. This code has been described in detail in the literature (2). The main features are shortly presented in the following.

The basic input source of the code consists of a table of numbers, τ/μ , representing the probabilities that a photon of energy E will produce a photoelectron instead of being Compton scattered. It is assumed that the cross section

for photoelectric effect is negligible for photon energies above 175 keV. Based on this table, and using standard Monte Carlo techniques, a decision is made for each individual photon as to whether it Compton scatters or produces a photoelectron.

In the case of a photoelectric process, photons with energy larger than 532 eV (the threshold for the K-shell in oxygen) produce an electron of energy $T = E - 532$, and an additional Auger electron of 508 eV. For photons with energies below 532 eV a photoelectron of energy $T = E - 12$ eV is assumed to be produced from the L shell. Photons with energies below 12 eV are neglected.

The Compton electrons are generated according to the classical equation:

$$\frac{d\sigma}{dT} = \text{const} \left[2 + \frac{T}{E-T} \left(\frac{1}{\alpha^2} + \frac{E-T}{E} - \frac{2}{\alpha} \frac{E-T}{T} \right) \right] \frac{\text{cm}^2}{\text{keV electron}}$$

where T is the energy of the electron, E is the energy of the photon and α is the energy of the photon measured in units of the electron rest mass (511 keV).

Following Compton scattering a secondary photon of energy $E-T$ appears. This photon is treated similarly to the initial photons. Again, the selection of the energy T is made using conventional Monte Carlo techniques. The calculation ends when all the photons (initial and secondary) are converted to electrons.

The initial energy spectrum of electrons calculated with this program for I-125 photons is shown in Fig. 1. Table 1 presents the mean energies of the photons considered and their mean number per disintegration.

The dose energy distribution, relevant for the calculation of the proximity function (see the previous section), is shown in Fig. 2. As expected the maximum dose contribution comes from 27 keV electrons.

The Simulation of Electron Tracks

Several Monte Carlo codes are presently in use for generating electron

Figure 1. The initial energy spectrum of electrons produced by I-125 photons in water calculated with the code PHOEL.

TABLE I

Mean Energy (keV)	Mean Number/Disintegration
35.3	0.0678
27.5	0.738
27.2	0.378
31.0	0.199
31.8	0.0413

Figure 2. The dose energy distribution corresponding to the spectrum of Figure 1.

tracks in various media (3-5). However, to date none of these programs were made available for distribution to outside users. It was necessary, therefore, to develop independently in our laboratory, a similar capability.

The transport formalism for the present code was taken from the paper by Grosswendt and Weibel (6). A Monte Carlo program was written based on this formalism. The reader should consult Ref. 6 (and references therein) for a complete and detailed description of the theoretical equations. A brief outline follows:

Three types of processes are allowed for the interaction of the electron with the material: elastic scattering, ionization and excitation. Following ionization a secondary electron is produced; this electron is followed in a manner similar to the primary particles.

The elastic scattering of an electron in the field of an atom is calculated using the Rutherford equation with a screening correction:

$$d\sigma_{el} = \frac{2\pi e^4}{(4\pi\epsilon_0)^2} \frac{z(z+1)}{4p^2 v^2} \frac{\sin\theta d\theta}{(\sin^2 \frac{\theta}{2} + \eta)^2}$$

with

$$\frac{e^4}{(4\pi\epsilon_0)^2} = 2.073 \times 10^{-26} \text{ cm}^2 \text{ MeV}^2$$

Here p is the momentum of the electron, v its velocity, z the atomic number of the medium, η a screening parameters, and θ the polar scattering angle.

The total elastic cross section is:

$$\sigma_{el} = 6.512 \frac{z(z+1)}{\eta(1+\eta)} \left[\frac{T + m_0 c^2}{T(T + 2m_0 c^2)} \right]^2 \times 10^{-26} \text{ cm}^2$$

where $m_0 c^2$ is the electron rest mass and the numerical constants correspond to energy measured in units of MeV.

Although theoretical predictions for the screening parameter η are available, Grosswendt and Weibel (6) suggest an empirical equation obtained from matching the above equations to measured cross sections.

The ionization of atoms and molecules is described using the analytical functions of Green and Stolarski (7). This process results in secondary electrons with energies less or equal to $(T-I)/2$ where I is the ionization energy (as a matter of convention the primary electron is considered always the particle with higher energy).

If k is the energy of the secondary electron, the angular distribution of the emitted electrons can be calculated using simple kinematics:

$$\sin^2 \theta_p = \frac{k/T}{(1-k/T) T/2m_0c^2 + 1}$$

$$\sin^2 \theta_s = \frac{1 - k/T}{1 + k / 2m_0c^2}$$

These equations are valid for energies higher than about 200 eV. For lower energies (down to about 50 eV) most of the secondary electrons are emitted at angles between 45° and 90° . Finally, below 50 eV, secondary electrons are emitted isotropically. Low energy primary electrons ($T < 100$ eV) are assumed to be emitted uniformly between 0 and 45° .

The excitation of atoms and molecules by electrons has been described analytically by Green and Stolarski (7). The cross section, $\sigma_{exc}^j(T)$ corresponding to an excitation level j , can be written:

$$\sigma_{exc}^j(T) = \frac{q_0 A}{k_j^2} \left(\frac{k_j}{T} \right)^\Omega \left[1 - \left(\frac{k_j}{T} \right)^\gamma \right]^\nu$$

where the parameters q_0 , k_j , A , Ω , ν and γ depend on the excitation state j and are tabulated for several gases. No changes in direction is assumed following excitation impact.

For each electron of energy T , the path length to the next interaction is simulated using the total cross section corresponding to that energy. The type of interaction (elastic, ionization or excitation) is selected with probability proportional to the respective partial cross sections. An interaction results in a change in direction and/or an energy deposition. For each interaction point, the position, energy, and the interaction type are recorded. Second - or higher - generation electrons are followed in an analog fashion. An arbitrary lower limit for the electron energy is set: below this the energy is assumed to be imparted locally (in the present case this limit is 10 eV).

Results

Two main objectives were set for the first stage of this work:

- a) establishing a working set of computer codes to generate initial electron spectra for photon fields, electron tracks and proximity functions. In addition, a number of auxiliary programs were necessary for more specific tasks (e.g., calculating dose average lineal energies, etc.)
- b) understanding the computer logistic involved in storing and using large amounts of memory data and the running times involved.

Electron tracks have been generated for the energies corresponding to the spectrum of Fig. 1, from 1 to 35 keV in steps of 2 keV. The material used was molecular nitrogen. This particular gas was chosen, aside from its relative simplicity in terms of theoretical cross sections, in order to investigate the possibility of its use as a substitute for more tissue-like media. All the programs ran on a PDP 11/45 computer operating under the UNIX system. Typical running times were 1 minute/event to generate a 5 keV electron track (proportional to the electron energy) and 1.5 minutes/5 keV track to obtain the proximity function (proportional to the second power of the electron energy). The computer

storage memory was used at a rate of 12 kbytes/(5 keV track).

The present results correspond to averages over 20 tracks for energies between 1 and 15 keV and 10 tracks for higher energies. Memory space and especially running times were the main limitations in the number of tracks simulated.

In Fig. 3 (a,b,c) the proximity functions obtained for 1, 2, 3, and 5 keV respectively are compared with similar results obtained by Chmelevsky et al. (8) for water. The present results although of the same general shape, are systematically lower by 10-20%, with larger differences at higher energies. Two factors could account for these differences:

- a) The material (i.e., molecular nitrogen vs. water), and/or
- b) the limited number of tracks utilized in the present calculation. This latter point might be quite significant in view of an analysis performed by Chmelevsky et al (8) with respect to the statistical significance of the results vs. the number of events generated.

A further comparison, shown in Fig. 4 (a,b,c) involves values of the dose average lineal energy, y_D , obtained in the two calculations. The results are consistent with the previous remarks. The relatively large differences at small distances might be artificial, related to the binning of the proximity function in our procedure (1 nm/bin).

In Fig. 5 the proximity function for I-125 in molecular nitrogen is presented. Finally, $\bar{y}_D$ values obtained with this function are shown in Fig. 6.

In light of these first results, a number of tasks are contemplated for the immediate and more distant future:

- a) direct tests of the statistical significance of the present results.
- b) updating the programs for other materials, in particular water.

Figure 3. A comparison between proximity functions for 3 keV (panel a), 1 and 2 keV (panel b) and 5 keV electrons (panel c) as obtained in the present calculation (dashed curves) and by Chmelevsky et al. (8) (solid curves).

Figure 4. $\bar{y}_D$ values as a function of the site radius for 1 keV (panel a), 2 keV (panel b) and 5 keV electrons (panel c). Solid curves represent the calculations of Chmelevsky et al. (8) and dashed curves show the present calculations.

Figure 5: Proximity function for electrons produced by I-125 photons in N_2 . The insert shows the initial part of the proximity function.

Figure 6: $\bar{y}_D$ values as a function of the site diameter, calculated from the proximity function represented in Figure 5.

- c) extension of the present formalisms to particles other than electrons.
- d) further comparisons and cooperative efforts with other groups.
- e) biophysical applications of the calculations.

References

1. A.M. Kellerer and D. Chmelevsky, Concepts of Microdosimetry, III. Mean Values of the Microdosimetric Distributions, Rad. and Environm. Biophys. 12: 321-335, 1975.
2. J.E. Turner, J.T. Modelo, G.M.A.A. Sordi, R.N. Ham, and H.A. Wright, A Monte Carlo Code (PHOEL) for Generating Initial Energies of Photoelectrons and Compton Electrons Produced by Photons in Water, ORNL/TM-6515, 1978.
3. H.G. Paretzke, An appraisal of the relative importance for microbiology of the effects of slow electrons. In Proc. Fifth Symp. on Microdosimetry Verbania-Pallanza, Italy (J. Booz, H.G. Ebert and B.G.R. Smith, Eds). p. 41-58, Commission of the European Communities, Luxembourg, 1976.
4. M. Terrissol, J. Fourmenty, and J.P. Patau, Determination theorique des fonctions microdosimetriques pour des electrons de basse energie dans les gaz. In Proc. Fifth Sump on Microdosimetry, Verbania-Pallanza, Italy (J. Booz, H.G. Ebert and B.G.R. Smith, Eds.), p. 393-406, Commission of the European Communities, Luxembourg, 1976.
5. J. E. Turner, R.N. Hamm, H.A. Wright, R.H. Ritchie, and R. Katz, Calculated electron slowing-down spectra for liquid water irradiated by X and gamma rays, In Proc. Sixth Symp. on Microdosimetry, Brussels, Belgium (J. Booz and H. G. Ebert, Eds.), p. 375-383, Harwood, London, 1978.
6. B. Grosswendt and E. Weibel, Transport of low energy electrons in nitrogen and air, Nucl. Instr. and Meth. 155: 145-156 (1978).
7. A.E. S. Green and R.S. Stolarski, Analytic models of electron impact excitation cross sections, J. Atmospheric Phys. 34: 1703 (1972).
8. D. Chmelevsky, A.M. Kellerer, M. Terissol, and J.P. Patau, Proximity Functions for Electrons up to 10 keV, preprint.

BP-4 Saturation Effects for Sparsely Ionizing Particles

M. Zaider and H.H. Rossi

Introduction*

In the Theory of Dual Radiation Action (TDRA)(1) the average yield of lesions per cell is related to the absorbed dose by the following expression:

$$\bar{\epsilon}(D) = k (\bar{z}_D D + D^2) \quad (1)$$

where $\bar{z}_D$ is the dose-averaged specific energy and k is a constant. In the presence of saturation effects $\bar{z}_D$ in Eq.(1) is replaced with the quantity $\bar{z}_D^*$:

$$\bar{z}_D^* = z_0^2 \int_0^\infty [1 - e^{-(z/z_0)^2}] f_1(z) dz / \int_0^\infty z f_1(z) dz \quad (2)$$

The Eq.(1) with $\bar{z}_D$ replaced with $\bar{z}_D^*$ is an approximate formulation of the more basic expression, describing the saturation effect:

$$\bar{\epsilon}(D) = k z_0^2 \int_0^\infty [1 - e^{-(z/z_0)^2}] f(z;D) dz \quad (3)$$

which reflects the simple assumption that the effect probability approaches a maximum value at large values of z (i.e., of the order of z_0). In Eqs.(2) and (3) $f(z;D) dz$ is the probability of a deposit of specific energy between z and $z + dz$ in a microscopic cavity when the dose is D ; and $f_1(z) dz$ is the corresponding single event distribution in z .

The Eq.(3) and especially its simplified corollary, Eq.(2), are geared toward explaining one of the classical examples of the saturation effect, namely the well-known RBE vs. LET curves as obtained with track segment experiments by Barendsen (2) and Todd (3).

It is useful at this point to examine more closely the assumptions behind the Eq.(2). As stated in Ref. 1:

"...it is a reasonable simplification to apply the [saturation] correction only to the single event component of the effect. This is justified insofar as the correction is significant only for high

*It is assumed in this article that the reader is familiar with the concepts of the TDRA as developed in Ref. 1.

values of z , and in this case the single event action is dominant."

Indeed, for high-LET particles, within the range of normal doses, single event traversals (i.e., intratrack interactions) will account for most of the observed effects. For low-LET radiation, however, high z values are still possible, but in this case as an accumulation of many small energy depositions by independent events; the intertrack modality will dominate. While Eq.(2) should describe correctly saturation effects for high-LET particles no such effects can be accounted for in the case of large doses of low-LET radiation (indeed from Eq.(2) $\bar{z}_D^* = \bar{z}_D$ in this case.) In fact, theoretical predictions based on the Eq.(1) tend to overestimate the amount of effect at large doses as observed in experiments, in agreement with the above observations.

The Eq.(3) cannot be solved exactly. In the present article an approximate solution of this equation is developed, valid under the conditions of low-LET radiation. This solution should be seen as complementary, rather than opposite to the formulation of Eq.(2).

Formal Treatment

The probability distribution $f(z;D)$ can be expressed formally as a compound-Poisson process:

$$f(z;D) = \sum_{v=0}^{\infty} e^{-n} \frac{n^v}{v!} f_v(z) \quad (4)$$

Here n is the average number of energy deposition events in the cavity (dependent on the dose D) and $f_v(z)$ is the probability to deposit the specific energy z in exactly v events. The physical interpretation of the Eq.(4) has been discussed extensively in the literature (1, 4); it is shortly repeated here for clarity. Energy deposition events are statistically independent. Therefore, the probability of exactly v events is given by the Poisson law. Further, the distributions $f_v(z)$ can be all derived by convolution from the single event spectrum $f_1(z)$:

$$f_v(z) = \int_0^z f_v - 1 (z - z') f_1(z') dz' \quad (5)$$

with

$$f_0(z) = \delta(z) \quad (6)$$

The first two moments and the variance of the distribution $f(z;D)$ can be readily calculated (1, 4):

$$\bar{z} = \int z f(z;D) dz = D \quad (7)$$

$$\bar{z}^2 = \int z^2 f(z;D) dz = \bar{z}_D D + D^2 \quad (8)$$

$$\sigma^2 = \bar{z}_D + D \quad (9)$$

where

$$\bar{z}_D = \int z^2 f_1(z) dz / \int z f_1(z) dz = \frac{\bar{z}^2}{\bar{z}_F} \quad (10)$$

and with an obvious definition for the single-event frequency average of z , called $\bar{z}_F$.

Finally, the average number of events, n , is given by:

$$n = D / \bar{z}_F \quad (11)$$

In general, the distribution $f(z;D)$ cannot be calculated in closed form and numerical procedures are necessary. However, in the limit of very large average numbers of events ($n \gg 1$) the distribution becomes gaussian, regardless of the shape of the single event distribution. This is precisely the region of interest for the present considerations.

Indeed, for a typical low-LET radiation (say 250 kv x-rays) and for a site diameter of 1 μm (characteristic for the biological end points described in the TDRA) one has (5):

$$\bar{z}_F \approx 25 - 38 \text{ rad} \quad z_0 = 2550 \text{ rad} \quad (12)$$

and for doses approaching the saturation region (z_0), n is of the order of 50 to 70. Under these conditions, using the Eqs.(7) and (8) one can write:

$$f(z;D) \approx \frac{1}{\sqrt{2\pi\bar{z}_D D}} \exp\left[-\frac{(z-D)^2}{2\bar{z}_D D}\right] \quad (13)$$

The Eq.(3) can now be integrated (the lower limit in the integral can be safely extended to $-\infty$, for convenience). The result is:

$$\bar{\epsilon}(D) = k z_0^2 \left[1 - \frac{1}{\sqrt{1+2\frac{\bar{z}_D}{z_0^2} D}} \exp\left(-\frac{1}{2\frac{\bar{z}_D}{z_0^2} D}\right) \right] \quad (14)$$

The Eq.(14) describes the average yield of lesions for doses sufficiently large to justify the approximation of Eq.(13) and taking into account the saturation effect. Although in the derivation of the Eq.(14) no discrimination need to be made between low- and high-LET radiations, the practical application of this equation is limited to low-LET fields for which the normality condition, $n \gg 1$, can be attained within the range of biologically significant doses.

A further useful approximation results if one considers the situation where, the Eq.(13) being still valid, one can assume:

$$2 \frac{\bar{z}_D}{z_0^2} D \ll 1 \quad (15)$$

As an example, for Co^{60} one has with site diameter $1 \mu\text{m}$ (5):

$$\bar{z}_F = 5.7 \text{ rad} \quad \bar{z}_D = 32.4 \text{ rad}$$

and for $D = 500 \text{ rad}$, $2 \bar{z}_D D / z_0^2 = 0.005$, $n = 88$.

Expanding the Eq.(14) around $D = 0$ and retaining only the first two powers of the dose, one obtains:

$$\bar{\epsilon}(D) \approx \alpha D + \frac{\beta}{1+2\frac{\alpha}{\beta}\frac{D}{z_0^2}} D^2 \quad (16)$$

where the notations $\alpha = k\bar{z}_D$, $\beta = k$, usually preferred in biological studies, have been used. In the limit of very low doses the Eq.(16) becomes the classical

expression with no saturation effects:

$$\bar{\epsilon}(D) = \alpha D + \beta D^2 \quad (17)$$

This last result is quite remarkable indeed: starting with a gaussian distribution, $f(z;D)$, valid for $n \gg 1$ only, one still obtains the correct expression by extrapolating back to low doses ($n \lesssim 1$) where the shape of $f(z;D)$ is in general strongly non-gaussian. The explanation is in fact quite simple: the result of Eq.(17), or its equivalent Eq.(1), depends only on the first and second moment of the single event distribution, $f_1(z)$. As shown in the Appendix, the gaussian function $f(z;D)$, Eq.(13), can be thought of, mathematically at least, as resulting from a fictitious single-event function, $f_1^*(z)$, having first and second moments only, and which are exactly equal to the respective moments of the real function, $f_1(z)$.

For high-LET particles, within a normal range of doses, one should still use the Eq.(1) modified according to the Eq.(2).

Example: Application to cellular inactivation

In this section the saturation formalism of the Eq.(14) is applied to the calculation of survival fractions, S , using the expression:

$$S(D) = d^{-\bar{\epsilon}(D)} \quad (18)$$

Figure 1 shows a set of experimental surviving fractions obtained by Schneider and Whitmore (6) after irradiating Chinese hamster embryonic fibroblasts (H1) with 280 kVp x-rays. This particular experiment was chosen because of the very low surviving fractions measured (4×10^{-5}). The theoretical curves in Fig. 1 (to be described below) are obtained by using a non-linear least square fitting procedure. Because of the fact that no statistical information on the experimental points was available, the goodness of the fit was estimated in each case by visual inspection.

The curve labeled A in Fig. 1 was calculated by fitting the Eq.(17) to the

Figure 1. Experimental surviving fractions for Chinese hamster embryonic fibroblasts irradiated with 280 kVp x-rays (Ref. 6). The theoretical curves are explained in the text.

whole set of data, with α and β as free parameters. The surviving fractions corresponding to low doses ($D < 600$ rad) are clearly underestimated. As an alternate, more realistic procedure, the expression of Eq.(17) was fitted to the first six dose data points (curve B). This is the range of doses where one indeed expects the Eq.(1) to be valid. Extrapolation to higher doses shows clearly the effects of saturation discussed in the previous sections.

Finally, the saturation-corrected expression of Eq.(14) was used: in this calculation (curve C), the values of α and β (or k and z_D) were taken from the previous curve (B), and the saturation constant, z_0 , was allowed as a free fitting parameter. Figure 1 shows that the saturation model of Eq.(14) describes well the experimental data. It is interesting to remark (within the limits of this particular example) that the best value obtained for the saturation parameter ($z_0 = 2427$ rad) corresponds closely to the value found previously for high-LET radiation (if one assumes a site diameter of $1 \mu\text{m}$).

REFERENCES

1. A.M. Kellerer and H.H. Rossi, The Theory of Dual Radiation Action. Curr. Topics Rad. Res. Q, 8: 85-158 (1972).
2. G.W. Barendsen, In: Theoretical and Experimental Biophysics, Ed. Cole, A. (Marcel Dekker, New York) 1: 167 (1967).
3. P.W. Todd, Reversible and irreversible effects of ionizing radiations on the reproductive integrity of mammalian cells cultured in vitro, Thesis, University of California, Lawrence Rad. Lab. UCRL 11614 (1964).
4. A.M. Kellerer, In: Microdosimetry, Proceedings 2nd Symposium on Microdosimetry, Ed. H.G. Ebert, Euratom, 4452 d-f-e, Brussels (1963).
5. J. Booz, Mapping of fast neutrons radiation quality, paper presented at the 3rd Symposium on Neutron Dosimetry in Biology and Medicine, Neuherberg, May 23-27, 1977.
6. D.O. Schneider and G.F. Whitmore, Comparative effects of neutrons and x-rays on mammalian cells, Rad. Res. 18: 286-306 (1963).
7. R.B. Bracewell, The Fourier Transform and its Applications. McGraw-Hill Book Company (1978).

APPENDIX

Let $\phi(\omega)$ and $\phi_1(\omega)$ be the Fourier transforms of $f(z;D)$ and $f_1(z)$, respectively. Following the convolution theorem (7), the Fourier transform of $f_v(z)$ is $\phi_1^v(\omega)$. The expression (4) can be written in frequency space as:

$$\phi(\omega) = \sum_{v=0}^{\infty} e^{-n} \frac{n^v}{v!} \phi_1^v(\omega)$$

which is the equivalent to:

$$\phi(\omega) = e^{n[\phi_1(\omega) - 1]} \quad (19)$$

This equation has been obtained previously by Kellerer (4). Let us further expand $\phi_1(\omega)$ around $\omega = 0$:

$$\phi_1(\omega) = \phi_1(0) + \phi_1'(0) \frac{\omega}{1!} + \phi_1''(0) \frac{\omega^2}{2!} + \dots \quad (20)$$

From the general formula (7):

$$\frac{d^n \phi_1}{d\omega^n} (\omega = 0) = (-2\pi i)^n \int_{-\infty}^{\infty} z^n f_1(z) dz$$

one has:

$$\begin{aligned} \phi_1(0) &= 1 \\ \phi_1'(0) &= -2\pi i \bar{z} \\ \phi_1''(0) &= -4\pi^2 \bar{z}^2 \end{aligned} \quad (21)$$

If one assumes that $f_1(z)$ has only the first three moments different from zero one has:

$$\phi(\omega) = \exp [-2\pi i n \bar{z} \omega - 2\pi^2 n \bar{z}^2 \omega^2] \quad (22)$$

The expression of Eq.(22) becomes in real space:

$$f(z;D) = \frac{1}{\sqrt{2\pi n \bar{z}^2}} \exp \left[- \frac{(z - n\bar{z})^2}{2n\bar{z}^2} \right] \quad (23)$$

which is equivalent to the Eq.(13), using Eqs.(7), (8), (9) and (11).

The derivation of Eq.(23) justifies and explains the result of Eq.(17).

BP-5 Sequential Exposures to Mixed Fields of Radiation. A Theoretical Approach

M. Zaider and H.H. Rossi

In the past several years a number of experiments have been reported (1-7) in which mammalian cells were sequentially exposed to mixed fields of radiation. Typically, in such experiments one measures cell survival using high-LET (first dose) followed after a varying time interval by a second dose of low-LET radiation.

The results of these experiments show that:

- a) The shape of the survival curve of the low-LET radiation changes as a function of the amount of initial high-LET dose delivered, and
- b) A recovery effect is present when the two radiations are delivered separated by a time interval.

Both these observations can be accounted for, qualitatively and quantitatively, within the framework of the Theory of Dual Radiation Action.

The basic assumption of the TDRA is that ionizing radiations produce entities termed sublesions, at a rate which is proportional to the specific energy, z . The yield of sublesions is thus cz where c is a constant. These sublesions interact pairwise to form lesions. If the probability of interaction between two sublesions is denoted by A , the yield of lesions is:

$$\epsilon(z) = Ac^2 z^2 = kz^2 \quad (1)$$

where $k = Ac^2$. The specific energy z is a stochastic quantity and for an absorbed dose, D , one can calculate the average number of lesions:

$$\epsilon(D) = \int \epsilon(z) f(z;D) dz \quad (2)$$

Here, $f(z;D) dz$ is the dose-dependent probability that the specific energy lies between z and $z + dz$, and is defined such that

$$\int f(z;D) dz = 1 \quad \int z \cdot f(z;D) dz = D \quad (3)$$

It has been shown (8) that $\epsilon(D)$ can be expressed as:

$$\epsilon(D) = k (\zeta D + D^2) \quad (4)$$

where ζ is the dose mean of the specific energy deposited in single events. In a more exact formulation (9) ζ is replaced by ξ which is a more complex quantity. However $\epsilon(D)$ remains proportional to two terms that are linear and quadratic in dose. The more general relation need therefore not be employed here. If one uses the simple assumption that each increment in the number of lesions eliminates a proportionate fraction of the cells able to form colonies (10), survival fractions can be calculated from the expression:

$$S = e^{-\alpha D - \beta D^2} \quad (5)$$

where, by choosing an appropriate constant k one has:

$$\alpha = k\zeta \quad \beta = k \quad (6)$$

The expression (5) has been extensively used to calculate survival fractions.

Consider now a sequential exposure with two doses D_1 and D_2 with the subscripts referring - here and in the following - to the two radiations. Let us neglect for the moment any recovery effects. The expression (1) can be rewritten for this case as follows: The specific energies z_1 and z_2 contributed by the two radiations produce $c_1 z_1$ and $c_2 z_2$ sublesions respectively. The yield of lesions is then:

$$\epsilon(z_1, z_2) = A(c_1 z_1 + c_2 z_2)^2 = (\sqrt{k_1} z_1 + \sqrt{k_2} z_2)^2 \quad (7)$$

$$\epsilon(D_1, D_2) = \iint \epsilon(z_1, z_2) f(z_1, z_2; D_1, D_2) dz_1 dz_2 \quad (8)$$

with obvious notations for k_1 and k_2 . Since z_1 and z_2 are *independent*

stochastic variables one can write:

$$f(z_1, z_2; D_1, D_2) = f_1(z_1; D) \cdot f_2(z_2; D_2) \quad (9)$$

Under these conditions using Eqs. (3) and (4) one obtains:

$$\varepsilon(D_1, D_2) = k_1 z_1 D_1 + k_2 z_2 D_2 + (\sqrt{k_1} D_1 + \sqrt{k_2} D_2)^2 \quad (10)$$

and one can express the survival fractions as:

$$S = \exp[-(\alpha_1 D_1 + \beta_1 D_1^2 + \alpha_2 D_2 + \beta_2 D_2^2 + 2\sqrt{\beta_1 \beta_2} D_1 D_2)] \quad (11)$$

This is the basic equation describing survival curves for sequential irradiation of cells with two different types of radiations. The expression (11) clearly describes the synergistic effect of sequential exposures:

The interference term $2\sqrt{\beta_1 \beta_2} D_1 D_2$ reflects the interaction between sub-lesions produced by the two types of radiation. In the case where both radiations have high LET it will usually be found that in the dose range of interest αD is much larger than βD^2 and it will be quite difficult to detect the interference term.

It should be noted that this treatment of the sequential exposures implies that the order in which the two radiations are applied is immaterial.

Fig. 1 shows examples of survival curves calculated using the equation (11) for $\alpha_1 = 0.95 \text{ Gy}^{-1}$, $\beta_1 = 0.012 \text{ Gy}^{-2}$, $\alpha_2 = 0.18 \text{ Gy}^{-1}$, and $\beta_2 = 0.04 \text{ Gy}^{-2}$. In each case, a first dose of the high-LET radiation is followed by graded doses of the low-LET one. Survival curves corresponding to independent effects are also shown for comparison. It is apparent from Fig. 1 that as one increases the initial high-LET dose, the characteristic "shoulder" for the low-LET curve tends to disappear. In other words, the lethal efficacy of the low-LET radiation increases as the dose of high-LET radiation is increased. These results are in agreement with observations made by a

Figure 1: Hypothetical calculated survival curves for high-LET radiation, low-LET radiation, and sequential exposures to a first dose of high-LET radiation followed by graded low-LET doses. The dashed curves indicate survival curves resulting from independent effects.

Figure 2: Experimental (7) and calculated survival fractions for sequential exposures of Chinese hamster V79 cells with deuterons (50 keV/ μ m) and/or 250 kVp x-rays. For details of the calculations see the text.

number of investigators (See Ref. 6). Fig. 2 shows the results of a recent experiment (7) in which Chinese hamster V-79 cells, synchronized in late S phase, have been irradiated separately or sequentially with deuterons (50 keV/ μm) and 250 kVp X-rays. The theoretical curves were obtained by fitting the Eq. (5) to the deuteron and X-ray data and using the Eq. (11) to calculate the shape of the combined irradiation curve. The agreement between the curve predicted by the present model and the experimental results is excellent.

In the following the problem of sublethal damage recovery will be considered. In the original development of the TDRA the following expression described the yield of lesions when sublesion damage is subject to time dependent repair

$$\epsilon(D) = k [\zeta D + q(t_1) D^2] \quad (12)$$

where t_1 is the time interval in which the irradiation occurs, and:

$$q(t_1) = \int_0^{t_1} \tau(t) h(t) dt \quad (13)$$

$\tau(t)$ describes the recovery process as a function of time, and $h(t)$ is the distribution of time intervals t between dose increments for a given temporal mode of irradiation (see Ref. 8). The reduction factor, $q(t)$, affects only the quadratic component, consistent with its interpretation as the yield of intertrack interactions.

For a constant dose-rate and assuming the exponential recovery function:

$$\tau(t) = e^{-t/t_0} \quad (14)$$

one obtains:

$$q(t_1) = \frac{2t_0}{t_1} - \frac{2t_0^2}{t_1^2} (1 - e^{-t_1/t_0}) \quad (15)$$

A similar formula can be derived for the case of sequential doses

of different radiations (see Ref. 11). If t_1 and t_2 are the respective time intervals in which two doses D_1 and D_2 are delivered, and if T is the time between irradiations (end of first dose to beginning of the second dose) one has:

$$S = \exp[-(\alpha_1 D_1 + \alpha_2 D_2 + q(t_1)\beta_1 D_1^2 + q(t_2)\beta_2 D_2^2 + 2q_{12}(t_1, t_2, T)\sqrt{\beta_1 \beta_2} D_1 D_2)] \quad (16)$$

where:

$$q_{12}(t_1, t_2, T) = \frac{t_0^2}{t_1 t_2} e^{-T/t_0} (1 - e^{-t_1/t_0}) (1 - e^{-t_2/t_0}) \quad (17)$$

(see Ref. 11). and $q(t_1)$, $q(t_2)$ are given by Eq. (15). The exponential recovery function, Eq. (14), was assumed in this calculation.

If the irradiation times, t_1 , t_2 are very short compared with t_0 , one obtains:

$$S = \exp[-(\alpha_1 D_1 + \alpha_2 D_2 + \beta_1 D_1^2 + \beta_2 D_2^2 + 2e^{-T/t_0} \sqrt{\beta_1 \beta_2} D_1 D_2)] \quad (18)$$

Fig. 3 shows different representations of the survival curves, Eq. (18), as a function of the time intervals between the two irradiations. As expected from the functional form of Eq. (18), the effect of repair becomes increasingly important as one delivers a larger initial high-LET dose. This last feature is again in agreement with experimental data (see Ref. 6).

REFERENCES

1. R. Railton, R.C. Lawson, and D. Porter, Interaction of γ -ray and neutron effects on the proliferative capacity of Chinese hamster cells. *Int. J. Radiat. Biol.* 27, 75-82 (1975).
2. R.E. Durand, and P.L. Oliver, Irradiation of multi-cell spheroids with fast neutrons versus X-rays: A qualitative difference in sub-lethal damage repair capacity or kinetics. *Int. J. Radiat. Biol.* 30, 589-592 (1976).

Figure 3. Calculated survival curves after exposure to a high-LET dose followed by graded low-LET doses as a function of the time between the two irradiations (expressed in units of t_0 , the recovery parameter of Eq. (14)).

3. F.Q.H. Ngo, A. Hun, and M.M. Elkind, On the repair of sub-lethal damage in V79 Chinese hamster cells resulting from irradiation with fast neutrons or fast neutrons combined with X-rays. *Int. J. Radiat. Biol.* 32, 507-511 (1977).
4. F.Q.H. Ngo, A. Hun, H. Utsumi, and M.M. Elking, Comparative radiobiology of fast neutrons: relevance to radiotherapy and basic studies. *Int. J. Rad. Onc. Biol. Phys.* 3, 187-193 (1977).
5. S. Hornsey, U. Andreozzi, and P.R. Warren, Sublethal damage in cells of the mouse gut after mixed treatment with X rays and fast neutrons. *Br. J. Radiol.* 50, 513-517 (1977).
6. F.Q.H. Ngo, E.A. Blakely, and C.A. Tobias, Do sublethal lesions and repair occur after high-LET radiation? Report LBL-7454, Lawrence Berkeley Laboratory, University of California.
7. R.P. Bird, Sequential exposures with high and low-LET radiations. (in preparation).
8. A.M. Kellerer, and H.H. Rossi, The Theory of Dual Radiation Action. *Curr. Top. Radiat. Res. Q.* 8, 85-158 (1972).
9. A.M. Kellerer and H.H. Rossi, A generalized formulation of dual radiation action. *Radiat. Res.* 75, 471-488 (1978).
10. H.H. Rossi and A.M. Kellerer, Biological implications of microdosimetry: I. Temporal aspects. *Proceedings of the Fourth Symposium on Microdosimetry, Verbania-Pallanza (Italy)*, p. 315 (1973).
11. M. Zaider and H.H. Rossi, The synergistic effect of different radiations. Accepted for publication in *Radiat. Res.* (1980).

Marco Zaider and Paul J. Kliauga

As in the past years the computer activity in this laboratory was geared toward biophysical and radiobiological applications. Most of the available programs run on the Cancer Center/Institute for Cancer Research PDP 11/45 computer operating under the Unix System. Although a modified FORTRAN Compiler is available, its use is not encouraged by the management group of the computer and consequently, the codes have been written entirely in the C-language. The newly-acquired Tektronix 4051 graphics terminal has proven to be an extremely valuable addition to the laboratories' computer hardware peripherals. This terminal allows for on-site display of processed microdosimetric spectra with a high degree of versatility in choice of display modes. An example of such computer-produced graphs is shown in section PM-2 of this report. These spectra were obtained by photographing the screen of the 4051 terminal. In the future, the acquisition of a hard-copy unit attached to the terminal will provide capability of producing publication-quality hard copy from which prints or slides can be obtained directly.

Much of the software for the analysis of microdosimetric spectra has been developed during the last few years. However, much ongoing development in refinements and modifications is always required. In fact, each experiment has different software requirements, and it has been found advantageous to develop "libraries" of programs which are then used as building blocks to construct a program best-suited to the analysis of a given experiment. Such software development often requires much computer time, but yields great dividends in more efficient data analysis.

The following is a list of the main programs added to our computer library during the past year. Most of these programs were written in an interactive mode,

- i.e., a question-answer input procedure which should be self-explanatory.
- 1) delta: a Monte-Carlo code to generate electron tracks in molecular nitrogen
 - 2) gauss: generates numbers distributed normally
 - 3) histmk: histograms and plots data
 - 4) marques: a non-linear least square fitting program
 - 5) marqmixed: calculates survival ratios for mixed field irradiations
 - 6) mlm: a fitting procedure based on the maximum likelihood method
 - 7) pair: simulates photon pair production
 - 8) phoel: a Monte Carlo code to calculate initial electron energies produced by
low energy photons
 - 9) proxill: calculates the proximity function, $t(x)$. The input comes from the
program delta
 - 10) proxiltot: calculates proximity functions for a radiation with a spectrum
of initial energies
 - 11) scat: histograms and plots 2-dimensional scattergrams
 - 12) spar: calculates stopping powers and ranges of charged particles in gases
(not for electrons)
 - 13) ysubd: calculates does averaged lineal energies

RADIATION BIOLOGY

- RB-1 Oncogenic transformation of mouse C3H 10T $\frac{1}{2}$ cells exposed to 300 kVp x-rays or 35 MeV neutrons
Richard C. Miller, Eric J. Hall (in collaboration with Dr. P. Shapiro, Naval Research Laboratory, Washington, D.C.)
- RB-2 Oncogenic transformation *in vitro* produced by misonidazole
Richard C. Miller and Eric J. Hall
- RB-3 Retinoids and a tumor promoter modify radiation induced transformation but not sister chromatid exchanges in rodent cells
Richard C. Miller, Charles R. Geard, Robert S. Osmak, Melanie Rutledge-Freeman, and Carmia Borek
- RB-4 X-ray induced *in vitro* neoplastic transformation of human diploid cells
Carmia Borek
- RB-5 Liver cells in culture: A model for investigating the regulation of retinol-binding protein metabolism
Carmia Borek (in collaboration with Drs. J.E. Smith and D.S. Goodman, Department of Medicine)
- RB-6 The oxygen enhancement ratio for high energy neutrons
Eric J. Hall and Myles Astor
- RB-7 Hypoxia mediated drugs as radiosensitizers and cytotoxic agents
Eric J. Hall, Myles Astor, Barbara Alonso and Steven Abrams
- RB-8 *In vitro* studies with cis-platinum diamide dihydrochloride and exogenous polyamines
Laurie Roizin-Towle and Louis Capuano
- RB-9 The development and decay of thermotolerance
Michael L. Freeman
- RB-10 Sensitivity to x-rays and UV of cis platinum (II) sensitive V-79 cells
Michael L. Freeman
- RB-11 Evaluating the interaction of protease inhibitors with DNA in human cells
Carmia Borek (in collaboration with J.E. Cleaver, Laboratory of Radiobiology, University of California, San Francisco)
- RB-12 Effects of long contact times of misonidazole on attached Chinese hamster V-79 cells
Charles R. Geard and Melanie Rutledge-Freeman

- RB-13 Clastogenic effects of misonidazole on aerated and hypoxic cells
in vitro
Charles R. Geard, Louis E. Schwartz, and Melanie Rutledge-Freeman
- RB-14 Antipain and radiation effects on oncogenic transformation and
sister chromatid exchanges in syrian hamster embryo and mouse
CeH 10T $\frac{1}{2}$ cells
Charles R. Geard, Melanie Rutledge-Freeman, Richard C. Miller
and Carmia Borek
- RB-15 Interaction of high- and low-LET sublethal damage: Effect on
the reproductive capacity of cultured human cells
Paul S. Furcinitti
- RB-16 Relative biological effectiveness of photons from ^{125}I at low
dose rates
Paul Goldhagen, Michael Freeman, and Eric J. Hall
- RB-17 Sequential exposures with high- and low-LET radiations
Richard P. Bird
- RB-18 The associated ion experiment: The effect of proton triads on
cell survival
Richard P. Bird

RB-1 Oncogenic transformation of mouse C3H/10T $\frac{1}{2}$ cells exposed to 300

kVp x-rays or 35 MeV neutrons

R.C. Miller, E.J. Hall (in collaboration with Dr. P. Shapiro,
Naval Research Laboratory, Washington, D.C.)

Controversy over the effects of low doses of high LET radiation and increased public interest in the potential harmful effects of ionizing radiation has stirred interest in using methods other than cell killing to assess radiation damage. The recent isolation and characterization of cells sensitive to post-confluent loss of contact inhibition has provided a tool to examine the carcinogenic potential of ionizing radiation.

The characteristics of the C3H/10T $\frac{1}{2}$ cl 8 mouse fibroblast cell line was first reported by Reznikoff et al. (1) while working in Charles Heidelberger's laboratory. These cells exhibit good contact inhibition and cease cell division at confluency. However, exposure of cells to physical or chemical carcinogens causes a small proportion of cells to form dense, piled up foci of transformed, non-contact inhibited cells. X-ray dose response curves over a wide range of doses show an increased transformation incidence with increasing dose (2). Exposure of cells to neutrons also has been reported to induce transformation (3).

Cells were exposed to neutrons with an average energy of 15 MeV from the Naval Research Laboratory, Washington D.C. Relative biological effectiveness (RBE) for transformation induction and cell killing between neutrons and 300 kVp x-rays were examined in this study. In addition, the effects of exposing cells to single or two doses of neutron split by 5 hours was determined.

Methods and Materials

Cell culture

Cells were cultured and maintained as previously described (2). Briefly, stock cells between passages 8 and 15 were grown in Eagle's basal medium supplemented with 10% fetal bovine serum and antibiotics. Cells were plated into 25 cm² flasks (Falcon Co.) 18 hours prior to irradiation at densities that resulted in about 30 cells per flask for cell survival determinations and 250 cells for transformation studies. After overnight attachment, the flasks were filled with medium and placed in insulated containers.

Flasks in the neutron treatment group and half the controls were transported to the Naval Research Laboratory (NRL), Washington, D.C. and upon arrival were placed in 37°C incubators. Single and split dose treatment groups were kept in incubators except during neutron irradiation. At the completion of irradiation, flasks were put back in the insulated carriers and returned to New York for incubation.

Flasks with cells scheduled for x-irradiation remained in New York and received the same handling as those flasks transported to NRL. This included storage in insulated carriers and movement in and out of incubators for irradiation at approximately the same time as the cells at NRL were being irradiated.

Two weeks later cell survival was calculated from the flasks seeded at low cell density after fixing cells in 10% formalin and staining with Giemsa. Cells for the transformation study were re-fed with fresh medium bi-monthly until fixation and staining at about 5 weeks after irradiation. At that time non-contact inhibited cells had overgrown the confluent monolayer of normal cells so that they appeared as darkly staining foci. These transformed foci

were identified and typed according to the criteria established by Reznikoff et al. (4). Only types 2 and 3 transformed foci were scored as transformants.

The low frequency of x-ray or neutron induced transformations prevented irradiation over the entire range of doses (1.5 to 8 Gy for x-rays and .02 to 3 Gy for neutrons) within one experiment. However, comparison of the effects of x-rays and neutrons for RBE determinations and single and split irradiation treatments was done at each dose point as self-contained experiments in order to provide valid comparisons.

X-irradiation

Attached cells were irradiated at room temperature using a Siemens Stabilipan x-ray machine operating at 300 kVp and 12 mA with 0.2 mm Cu external filtration. The dose rate to the cells from measurements with a Victoreen R-meter embedded in a lucite phantom was calculated at 0.32 Gy min^{-1} .

Neutron irradiation

Notes on dosimetry for the cell transformation experiments

The neutron irradiations were performed at the Middle Atlantic Neutron Therapy Facility (5) (MANTA) utilizing the standard MANTA radiotherapy beam generated by bombarding a thick beryllium target with 35 MeV deuterons. This beam has a continuous neutron spectrum (6), with a peak at 14 MeV.

The Falcon tissue culture flasks to be irradiated were placed in the standard lucite phantom (6-8), adopted for radiological inter-comparisons between the various neutron therapy facilities. The front face of the phantom was placed at an SSD of 136.4 cm, and a collimator insert was used which produces at 15 cm x 15 cm field (90% width) at that SSD. The cells are plated on the surface of the flask that is downstream from the beam, the

tissue culture medium acting as build-up material so that the cells are deeper than the depth for transient charged particle equilibrium. The dose rate at the cells was approximately .45 Gy/minute.

The neutron dose in the plane of the cells was measured by substituting a lucite panel with a cutout for an ion chamber for the cell holders. The relative dose with respect to central axis is shown with the dose measurement being taken at the average dose position. This tissue-equivalent ion chamber, the EG&G (9) Model IC-17, 1-cm³ ion chamber, has a ⁶⁰Co calibration factor traceable to the National Bureau of Standards. The derivation of neutron sensitivity from the ⁶⁰Co sensitivity is fully discussed in Refs. 10 and 11. Intercomparisons between the various neutron therapy facilities show agreement in neutron dose measurement to better than 1.5%. No displacement correction has been applied to the in-phantom dosimetry because of the uncertainty in the displacement correction for this geometry. Errors due to lack of displacement correction will essentially cancel out in evaluation of RBE because the displacement correction is not applied to the photon dosimetry either.

Results

Survival data for cells after exposure to x-rays and neutrons were as expected. A characteristic broad shoulder followed by an exponential reduction in cell survival is evident in the curve for cells exposed to x-rays while a much smaller shoulder is seen for survival of neutron-irradiated cells. The resulting RBE at 0.1 survival is 2.8. The D_0 for x-irradiated cells is calculated to be 2.0 Gy and for neutron-irradiation the D_0 is 0.80 Gy.

A dose response relationship for x-ray and neutron induced transformation

frequency is presented in Fig. 1. Cells exposed to x-ray doses from 1.5 to 8 Gy show an increased linear induction with increasing dose. This is in agreement with previously published results (2).

Results similar to the cellular response observed with high doses of x-rays are seen for cells exposed to neutrons in the dose range from 0.5 to 3 Gy. The curve, however, is displaced to the left indicating increased cellular radiosensitivity and the slope appears less steep than for x-rays. At neutron doses between 0.02 and 0.5 Gy a shallow response curve is seen. In this dose range increasing doses results in only a slightly higher transformation frequency.

As a consequence the RBE value at high doses was calculated at 2.9 at 1.5×10^{-3} transformants/surviving cell and increased to 75 for low dose exposures where the transformation frequency is 1×10^{-4} .

Transformation frequencies of cells exposed to low doses of neutrons given either as a single dose or in two equal fractions separated by 5 hrs is shown in Fig. 2. Although, it is tempting to conclude that splitting doses of neutrons below 0.5 Gy results in the enhancement of transformations, statistically this has not been shown.

Discussion

At high doses of x-rays or neutrons the RBE for cell killing is similar to that for the induction of transformations. Lower doses of these two types of ionizing radiation result in larger RBEs for both endpoints mentioned. The RBE for cell killing, however, can not be calculated in the low dose shoulder region of the survival curve whereas transformation induction can be compared down to very low exposures. RBE values as high as 75 for transformation are reported here for doses of 0.02 Gy neutrons and 1.5 Gy x-rays.

Figure 1: Dose response of cells exposed to 300 kVp x-rays or 35 MeV neutrons.

Figure 2: Induction of transformation for cells exposed to a single dose of neutrons or given as two equal doses separated by 5 hours.

With the time interval of 5 hours and doses ranging from 0.02 Gy to 2.0 Gy, no statistically significant enhancement or sparing occurred when neutron doses were split as compared to a single exposure.

In light of the RBE values obtained for low doses of high LET radiation (compared to x-rays) protection guidelines can no longer be assumed to be conservative estimates of acceptable exposure to high LET radiation.

References

1. Reznikoff, C.A., Brankow, D.W., and Heidelberger, C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* 33:2121-2128 (1973).
2. Miller, R.C., Hall, E.J. and Rossi, H.H. Oncogenic transformation of mammalian cells *in vitro* with split doses of x-rays. *Proc. Natl. Acad. Sci. USA* 76:5755-5758 (1979).
3. Han, A. and Elkind, M.M. Transformation of mouse C3H/10T $\frac{1}{2}$ cells by single and fractionated doses of x-rays and fission-spectrum neutrons. *Cancer Res.* 39:123-130 (1979).
4. Reznikoff, C.A., Bertram, J.S., Brankow, D.W. and Heidelberger, C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* 33:3239-3249 (1973).
5. Theus, R.B., Bondelid, R.O., Attix, F.H., August, L.S., Shapiro, P. Surratt, R.E. and Rogers, C.C. *Cancer* 34:17 (1974).
6. August, L.S., Shapiro, P. and Theus, R.B. Neutron Spectral Measurements and Interpretation at the Naval Research Laboratory, in NBSIR 77-1279, Cross Sections and Yields for High Energy Neutron Source Reactions, July (1977).

7. Hall, E.J. Radiobiological intercomparison *in vivo* and *in vitro*.
Proceedings: Particles and Radiation Therapy Second International
Conference, J. Rad. Oncol., Biol. Phys. 3:195 (1977).
8. Hall, E.J., Withers, H.R., Geraci, J.P., Meyer, R.E., Rasey, J.,
Todd, P. and Sheline, G.E. Int. J. Rad. Oncol., Biol. Phys. 5:227
(1979).
9. EG&G, Inc. Goleta, CA 93017.
10. Attix, F.H., Theus, R.B., Shapiro, P., Surratt, R.E., Nash, A.E. and
Gorbics, S.G. Phys. Med. Biol. 16:497 (1973).
11. Smith, A.R., Almond, P.R., Smathers, J.B., Otte, V.A., Attix, F.H.,
Theus, R.B., Wootton, P., Bichsel, H., Eenmaa, J., Williams, D.,
Bewley, D.K., and Parnell, C.J. Med. Phys. 2:195 (1975).

RB-2 Oncogenic Transformations *in Vitro* Produced by Misonidazole

R.C. Miller and E.J. Hall

INTRODUCTION

Misonidazole has been shown to possess many of the properties required for a clinically useful radiosensitizer of hypoxic cells (1, 5). The use of the drug in radiotherapy patients is limited by the development of a peripheral neuropathy as well as symptoms of central nervous system damage that result from repeated applications of high doses. Because of these undesirable side-effects, a considerable effort has been made to synthesize alternative compounds that equal Misonidazole as radiosensitizers, while producing less neurological toxicity. A bewildering array of new compounds is now available, and the choice of the next one to try in the clinic is not obvious.

The reports that metronidazole induces mutations *in vitro* and multiple tumor types *in vivo*, while misonidazole induces mutations and oncogenic transformations *in vitro*, have raised questions concerning the possible adverse long term effects of these radiosensitizers (4, 7, 9). As radiosensitizers are used in younger patients with a more favorable prognosis, this will become a factor to consider. An additional parameter to consider in choosing the next radiosensitizer to test in clinical trials might be its oncogenic potential. If several compounds were available which were similar in radiosensitizing and cytotoxic properties, then the one with the lesser oncogenic potential would be the obvious choice.

The present report compares misonidazole with a number of other compounds that have been suggested as alternatives, using an established cell line to score oncogenic transformations *in vitro*. The four electron affinic compounds studied

were misonidazole (Ro-07-0582), desmethylnisonidazole (Ro-05-9963), Ro-07-0741 and SR 2508.

MATERIALS AND METHODS

The fraction of cells surviving the various drug treatments was determined by plating an appropriate number of cells into 100 mm Petri dishes, so that approximately 50 cells survived. The cells were fixed with formalin and stained with Giemsa 12 days after treatment, by which time the individual colonies could be counted readily to assess surviving fraction.

For transformation studies cells were plated at a density that allowed for approximately 400 reproductively viable cells per dish to survive the drug treatment. The drugs were added to the cells after overnight attachment for 18 to 24 hours, and remained in contact with the cells under aerated conditions for 3 days. Medium was then removed, replaced with drug-free medium, and changed weekly until the termination of the experiment 5 weeks later.

The criteria for identifying transformed colonies by their altered morphology as described by Reznikoff et al. (8) was used in this study. Only types 2 and 3 transformations were used in determining transformation frequency. Type 3 foci is shown in the photomicrograph in Figure 1. Transformation frequencies were calculated by dividing the number of transformed foci by the number of cells surviving the treatment. Confidence limits were determined assuming a Poisson distribution.

RESULTS

Survival data for aerated cells exposed for 3 days to misonidazole at concentrations from 0.4 to 8 mM are shown in Figure 2. After an initial shoulder, the fraction of cells surviving approximates to an exponential function of drug concentration. In parallel with the survival studies, the incidence of oncogenic

Figure 1. Type 3 transformed focus. Note the evenly spaced nontransformed monolayer of cells in the background which contrasts with the densely stained piled-up transformed cells.

Figure 2. Survival curve of aerated C₃H/10T $\frac{1}{2}$ cells exposed for 3 days to misonidazole. Survival was calculated as (number of observed colonies)/(number of cells plated x plating efficiency).

transformation was scored, and these data are shown in Figure 3. The frequency of transformants per surviving cell increases with drug concentration over the range tested.

Misonidazole was compared with desmethylnisonidazole, Ro-07-0741 and SR 2508 in a series of large self-contained experiments. The data are shown in Table 1. In all cases the drug treatment consisted of a concentration of 1 mM for 3 days. Misonidazole and desmethylnisonidazole induced similar frequency of transformation, while Ro-07-0741 produced twice as many transformants and SR 2508 five times as many.

DISCUSSION

The first aim of the experiments reported here was to compare the oncogenic potential of misonidazole with other drugs suggested as alternatives. The four drugs tested are virtually indistinguishable in radiosensitizing efficiency, but can be separated clearly on the basis of their ability to produce oncogenic transformations *in vitro*. Misonidazole and desmethylnisonidazole produce the fewest transformations, SR 2508 the most, with Ro-07-0741 approximately mid-way between.

The oncogenic potential of the radiosensitizers is compared with ionizing radiations, as well as with a range of commonly used chemotherapeutic agents in Table 2. The transformation frequencies for the chemotherapy agents were taken from three reports in the literature (3, 6, 10).

Two conclusions can be drawn from this comparison. First, most chemotherapeutic agents are vastly more potent than misonidazole in producing transformations *in vitro*. At the top of the list is cis-Platinum, though many other commonly used agents are not far behind. In the case of these chemotherapy agents, there is a good correlation between a high incidence of transformation *in vitro* and reports of carcinogenesis in patients. In view of the relatively

Figure 3. Percentage of transformants per surviving cell as a function of miconidazole concentration. Cells were exposed to the drug for 3 days.

Table 1

In Vitro Oncogenic Transformations of C3H/10T $\frac{1}{2}$ Cells Exposed to Hypoxic Cell Sensitizers

3-Day Exposure of 1 mM Drug	SF (PE)	Total Surviving Cells	Total No. of Transformed Foci	Frequency of Transformants per Surviving Cell $\pm$ 1 S.E.
Misonidazole (Ro-07-0582)	0.72	15.87×10^4	36	$2.27 \pm 0.38 \times 10^{-4}$
Ro-05-9963	0.74	14.63×10^4	40	$2.73 \pm 0.43 \times 10^{-4}$
Ro-07-0741	0.60	5.06×10^4	23	$4.55 \pm 0.95 \times 10^{-4}$
SR 2508	0.71	8.72×10^4	100	$11.47 \pm 1.15 \times 10^{-4}$
Control	(0.29)	4.45×10^4	0	0

Note: All values represent the average of two to four experiments. Standard errors were calculated by $1/(\text{No. of transformed foci})^{-1/2} \times (\text{transformation frequency})$.

Table 2

COMPARISON OF *in vitro* ONCOGENIC TRANSFORMATIONS WITH *in vivo* CARCINOGENESIS PRODUCED BY CHEMOTHERAPY AGENTS, RADIOSENSITIZERS, X-RAYS AND NEUTRONS

AGENT	Dose	Cell	<i>In Vitro</i> Transformation Incidence	<i>In Vivo</i> Carcinogenesis Demonstrated
<u>CHEMOTHERAPY AGENTS</u>		<u>μg/ml</u>		
Cis-Platinum (11)	0.5	fresh explant	12	?
Adriamycin	0.01	M2	1.7	+
Daunomycin	0.005	"	0.85	?
Actinomycin-D	0.005	"	0.074	?
Melphalan	1.0	10T $\frac{1}{2}$	2.94	+
Hycanthone	5.0	"	2.7	+
Actinomycin-D	0.1	"	1.92	+
Thiotepa	0.5	"	1.5	+
Bleomycin	2.5	"	0.5	+
5-Azacytidine	2.5	"	0.45	+
Methotrexate	100	"	0.16	+
Vincristine	0.05	"	0.09	-
6-Mercaptopurine	1000	"	0	-
Tilorone	5	"	0	-
<u>RADIOSENSITIZERS</u>		<u>mM</u>		
Misonidazole	1	10T $\frac{1}{2}$	0.023	?
Desmethylmisonidazole (9963)	1	"	0.027	?
Ro-07-0741	1	"	0.046	?
SR-2508	1	"	0.11	?
<u>RADIATION</u>		<u>rad</u>		
X-rays	100	10T $\frac{1}{2}$	0.025	+
Neutrons	100	"	0.060	+
	50	"	0.023	+

FOOTNOTE TO TABLE 2

1. Data for chemotherapy agents taken from Benedict et al. (2).
2. Chemotherapy drug doses approximate to the clinically observed peak concentrations.
3. The data for radiosensitizers refers to a 3-day exposure at 1 mM.

relatively short times for which many of these agents have been available, clinical reports of carcinogenesis are likely to be associated with extremely high levels.

The nitroimidazole sensitizers are also compared with x-rays in Table 2. A three day exposure to misonidazole or desmethylmisonidazole at a concentration of 1 mM results in an incidence of transformation comparable to about 100 rad of x-rays. Since the drug comes into contact with essentially all of the tissues of the body, the relevant comparison is with total body radiation exposure. Based on the analysis of the survivors of Hiroshima and Nagasaki, the total risk of cancer from exposure to 100 rad of x-rays is of the order of 1 in 100 (2, 11).

It must be concluded therefore that the carcinogenic potential of the new generation of radiosensitizers, while not negligible, is considerably less than that associated with many chemotherapeutic agents in common use. The various 2-nitroimidazoles tested, all of which have virtually identical radiosensitizing efficiencies, differ significantly in the rate at which they produce oncogenic transformations *in vitro*. This factor must be taken into account, in addition to properties such as the partition coefficient and the half-life *in vivo*, in choosing which of the many possible drugs is to be used in clinical trials as an alternative to misonidazole.

REFERENCES

1. Asquith, J.C., Watts, M.E., Patel, K., Smithen, C.E. and Adams, G.E.
Electron affinic sensitization v. radiosensitization of hypoxic bacteria and mammalian cells *in vitro* by some nitroimidazoles and nitropyrazoles.
Radiat. Res. 60:108-118, 1974.

2. BEIR Biological Effects of Ionizing Radiation. Report of the Advisory Committee, National Academy of Sciences, USA. National Research Council, Washington, D.C., 1972.
3. Benedict, W.F., Banerjee, A., Gardner, A. and Jones, P. Induction of morphological transformation in mouse C3H/10T $\frac{1}{2}$ clone 8 cells and chromosomal damage in hamster A(T $_1$)Cl-3 cells by cancer chemotherapeutic agents. Cancer Res. 37:2202-2208, 1977.
4. Connor, T.H., Stoeckel, M., Evrard, J. and Legator, M.S. The contribution of metronidazole and two metabolites to the mutagenic activity detected in urine of treated humans and mice. Cancer Res. 37:629-633, 1977.
5. Denekamp, J. and Harris, S.R. Tests of two electron-affinic radio-sensitizers *in vivo* using regrowth of an experimental carcinoma. Radiat. Res. 61:191-203, 1975.
6. Marquardt, H., Phillips, F.W. and Sternberg, S.S. Tumorigenicity *in vivo* and induction of malignant transformation and mutagenesis in cell cultures by Adriamycin and Duanomycin. Cancer Res. 36:2065-2069, 1976.
7. Miller, R.C. and Hall, E.J. Oncogenic transformation *in vitro* by the hypoxic cell sensitizer misonidazole. Br. J. Cancer 38:411-417, 1978.
8. Reznikoff, C.A., Brankow, D.W., Heidelberger, C. Quantitative studies of chemical transformation of C3H mouse embryo cells. Cancer Res. 33:3239-3249, 1973.
9. Rustia, M., Shubik, P., Patil, K., Clayson, D. Multiple tumor types induced with trichomonocide drug Flagyl in rats. Proc. Am. Assoc. Cancer Res. 20:54, abstract, 1979.

10. Turnbull, D., Popescu, N.C., DiPaolo, J.A. and Myhr, B.C. Cis-Platinum (II) diamine dichloride causes mutation, transformation, and sister-chromatid exchangers in cultured mammalian cells. *Mutat.Res.* 66:267-275, 1970.
11. UNSCEAR United Nations Scientific Committee on the Effects of Atomic Radiations Report to the General Assembly on Sources and Effects on Ionizing Radiations, 1977.

RB-3 Retinoids and a tumor promoter modify radiation induced transformation but not sister chromatid exchanges in rodent cells

R.C. Miller, C.R. Geard, R.S. Osmak, M. Rutledge-Freeman, C. Borek

Introduction

Vitamin A analogs (retinoids) (8) have been shown to reduce the effects of carcinogens both *in vivo* and *in vitro* (2, 6, 9) and this effect has stimulated considerable interest in their use in clinical applications (21). Conversely, a group of compounds derived mainly from plant materials have been shown to be potent enhancers of carcinogenesis *in vivo* and *in vitro*.

In recent years a number of laboratories have reported data from experiments *in vivo* and *in vitro* indicating that antagonism exists between retinoids and tumor promoters (9, 13, 23-25) or tumor promoter - like sarcoma factor (23). Some of these interactions have been reviewed (12). Tumor promoters and retinoid alone or in combination have been shown to have a multiplicity of actions on both neoplastic and normal cells. However, the effects of these agents on oncogenic transformation are poorly understood.

We have reported that a vitamin A analog inhibits radiation induced transformation in mouse C3H/10T $\frac{1}{2}$ Cl. 8 cells *in vitro* (6). Since TPA has been shown to enhance radiation induced transformation in C3H/10T $\frac{1}{2}$ cells (8), it was of interest to follow up on these various observations and to establish induced transformation *in vitro* and, if so, to examine whether the effects were reflected in patterns of DNA damage using sister chromatid exchange analysis.

Methods : Materials

Chemicals

The vitamin A analogs TMMP-ERA, TMMP-ERO, TMMP-RA and 13 RA were obtained from Hoffman La Roche of Nutley, N.J. β -RA was kindly donated by Dr. M. Sporn. Care was taken to avoid exposing the retinoids and cells containing BrdU to anything other than subdued natural or gold light.

TPA (Consolidated Midland Corp., Brewster, N.Y.) was prepared in DMSO prior to each experiment. The final concentration of TPA and DMSO in the growth medium was 0.16 μ M (0.1 μ g/ml) and 0.01% respectively.

Methods of irradiation

Attached cells were irradiated at room temperature using a Siemens Stabilipan x-ray machine operating at 300 kVp and 12 mA, with 0.2 mm Cu external filtration. The dose rate from measurements with a Victoreen R-meter at the location of the cells was calculated at 0.32 Gy min⁻¹.

Cell culture

a) Mouse C3H/10T' Cl. 8 cells. Cells were cultured and maintained as originally described by Reznikoff, et al. (20).

The abbreviations used are: TMMP-ERA, trimethylmethoxyphenyl analog of N-ethyl retinamide; TMMP-ERO, trimethylmethoxyphenyl analog of ethyl Retinoate, TMMP-RA, trimethylmethoxyphenyl analog of β retinoic acid; 13-RA, 13-cis retinoic acid; β -RA, β -all-trans retinoic acid; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; DMSO, dimethyl sulfoxide; BrdU, bromodeoxuridine; BP, benzo-a-pyrene. SCE, sister chromatid exchange. Gy min⁻¹, gray per minute.

Cell growth characteristics were determined by plating 4×10^4 cells per dish 18 hours prior to 4 Gy of x-rays and/or to exposure to retinoids and/or TPA. Cells were in contact with the retinoids and TPA throughout the testing period which included medium replacement every six days.

Transformation experiments involved using stock cells between passages 9 and 14. Cells were plated 18 hours prior to treatment and at densities that resulted in survival of about 40 clones per dish for determination of plating efficiency and cell survival. Cells were fixed in 10% formalin and stained with Giemsa 12 days after plating.

Concurrently, cells were plated for transformation studies into 50 cm^2 Petri dishes such that approximately 400 reproductively viable cells survived the treatments. In those cultures requiring retinoids, cells were exposed to the compound for 4 days. TPA where appropriate, was in contact with the cells throughout the cell incubation period. Cells in medium alone or medium plus TPA were subsequently re-fed weekly until termination of the experiment 5 weeks after plating when cells were fixed and stained with Giemsa. At that time transformed non-contact inhibited cells had overgrown the confluent monolayer of normal cells. The frequencies of darkly staining transformed foci indicating a loss of cell contact inhibition were recorded and assessed according to the criteria established by Reznikoff, et al. (19). Only Types 2 and 3 transformed clones were scored as transformations.

b) Primary Hamster Embryo Cells. Short term cultures of freshly explanted midterm hamster embryos were prepared as originally described (4).

Cells were seeded in 25 mm flasks (Falcon) at a concentration per flask of 10^3 cells seeded on 3×10^4 syngeneic feeder cells (3) in the presence of $7.1 \mu\text{M}$ β -RA. The cultures were irradiated 24 hours after seeding with 3 Gy of x-rays. TPA was added immediately after irradiation. β -RA

remained in the medium for 3 days following irradiation; after which medium free of retinoid, but containing TPA was used for cell feeding. Because Syrian hamster cells metabolize TPA (16) it was considered prudent to change the medium every 2 days. The first medium change contained both retinoid and TPA, but the subsequent changes contained TPA alone. Control plates consisted of cells, unirradiated retinoid treated cells, and unirradiated TPA treated cells.

The duration of the experiment was 10 days, after which cultures were fixed, stained, and scored for transformation using morphological criteria as described previously (4). Transformed colonies were scored from among the surviving colonies thus allowing the evaluation of plating efficiency, cell killing and cell transformation within the same experiment.

Sister chromatid exchanges

A number of treatment conditions were used during the course of these experiments, these are detailed in the results. In general, BrdU ($3 \mu\text{M}$) was made available to asynchronous log-phase cells for 24 to 26 hours before irradiation (3 Gy), TPA and/or retinoid. Cells were fixed at various times 10 to 20 hours after irradiation with mitoses being accumulated with colcemid for the final 6 hours. In each of 5 replicate experiments BP was available to the cells for the whole period to act as a positive indicator of sister chromatid exchange induction.

Cells were stained in 2% Giemsa (Gurrs R66) in $0.3 \text{ M Na}_2\text{HPO}_4$ at pH 10.4 (adjusted with 1 N NaOH) for 10 to 30 minutes (1). Cells that have been in BrdU for one, two or three replication cycles are readily assessed and the frequencies of each were recorded in mitotic cell samples of 200 from each treatment. The percentage of cells in mitosis (mitotic index) was also determined from samples of 1,000 cells from each treatment.

Sister chromatid exchanges were recorded on a per chromosome basis in samples of 1,000 to 2,000 chromosomes from each treatment from cells in their second metaphase after BrdU incorporation. Radiation-induced SCE's are thus induced in one cycle only, whereas the chemicals can induce SCE's in both cell cycles.

Results

Growth characteristics of mouse C3H/10T $\frac{1}{2}$ and Syrian hamster embryo cells exposed to x-rays, retinoids and TPA

The cell density in clones arising from TPA treated cells was greater than that of control cells, as shown in Figure 1. Appearances were similar for both C3H/10T $\frac{1}{2}$ and hamster embryo cells in all groups that were treated with TPA regardless of any additional exposure to x-rays or retinoid.

The growth of C3H/10T $\frac{1}{2}$ cells exposed to two concentrations of TMMP-ERA and TMMP-ERO are presented in Figure 2. Doubling time for cells in contact with TMMP-ERA at 7.1 μ M was similar to controls; while in 71 μ M of retinoid it was almost twice the control. Cell growth in TMMP-ERO showed an even more profound concentration dependence. Syrian hamster embryo cells showed no growth inhibition in the presence of 7.1 μ M β -RA.

Cells that were exposed to 7.1 μ M concentrations of two additional retinoids (TMMP-RA and 13-RA) showed a substantial inhibition of growth when compared to cells exposed to 7.1 μ M TMMP-ERA and TMMP-ERO. This growth inhibition was considered to be sufficiently severe as to preclude further studies with the TMMP-RA and 13-RA.

Cell doubling times for control and radiation transformed cells in drug-free medium were similar (Figure 3). However, while the non-transformed cells reached confluency and plateaued at 2.3×10^6 cells per 50 cm²

Figure 1. Photomicrographs of normal C3H/10T $\frac{1}{2}$ clones (A) and TPA treated clones (B) 12 days after plating. 300 cells derived from an asynchronous, log-phase population were plated in 100 mm dishes and TPA (0.16 μ M) added 6 hours later. The TPA remained in continuous contact with cells and resulted in denser colonies while not effecting plating efficiency or cell number. After 12 days growth cells were fixed and stained with Giemsa.

Figure 2. Growth characteristics of C3H/10T $\frac{1}{2}$ cells continuously exposed to two concentrations of the retinoids TMMP-ERA and TMMP-ERO. The growth inhibitory nature of the higher retinoid concentration is readily seen from these Coulter counts of total cell numbers after trypsinisation from 100 mm cell dishes.

Figure 3. Growth characteristics of normal and radiation-transformed C3H/10T_{1/2} cells in the presence and absence of the retinoid TMMP-ERA. Cells were in contact with the retinoid throughout and were counted in a Coulter counter after trypsinisation from 100 mm cell dishes.

dish, the transformed cells continued to grow beyond confluency and reached a density of 4×10^6 cells per dish at 13 days. Transformed cells in retinoid, however, showed inhibition of cell growth compared to normal cells in retinoid.

The growth curves for cell exposure to single or combined treatments of TMMP-ERA, TPA and 4 Gy x-rays are presented in Figure 4. Doubling times were similar to controls for cells treated with $0.16 \mu\text{M}$ TPA, $7.1 \mu\text{M}$ TMMP-ERA or both combined. However, it is important to note that the treatment groups that contained TPA (TPA alone, TPA plus retinoid) reached confluency with substantially greater cell densities than control or retinoid.

Cells exposed to x-rays alone or in combination with retinoid and/or TPA showed similar lesser growth rates than untreated controls. Although irradiation initially inhibited the growth rate of cells, it was the presence or absence of TPA that determined final cell density.

Survival of C3H/10T $\frac{1}{2}$ and Syrian hamster embryo cells after treatment with 4 Gy x-rays, TPA, or retinoid

C3H/10T $\frac{1}{2}$ cells exposed to TPA showed little cell death; the retinoid TMMP-ERA showed some cytotoxicity, and 4 Gy of x-rays reduced cell survival to 31% (Table 1). Qualitatively, exposure of hamster embryo cells to the above treatments resulted in cell survival similar to the C3H/10T $\frac{1}{2}$ cells (Table 2). The combination of irradiation and TPA resulted in enhanced cell survival compared to irradiation alone for both rodent cell lines.

Modification of transformations in C3H/10T $\frac{1}{2}$ and Syrian hamster embryo cells

Exposure of cells to TPA alone, retinoid alone, or the combination of the two compounds produced no transformations (Tables 1 & 2). However, addition of TPA immediately followed by continuous exposure of cells to

Figure 4. Growth characteristics of C3H/10T $\frac{1}{2}$ cells exposed to the combination of 4 Gy x-rays, TMMP-ERA and TPA. Cells were in contact with the retinoid and TPA throughout. Cell numbers were counted in a Coulter counter after trypsinisation from 100 mm cell dishes.

Table 1. The Modulation of X-ray Induced Cell Transformation by TPA and Retinoid TMMP-ERA in the C3H/10T $\frac{1}{2}$ Cell Line

Treatment	SF(PE) ^a	Total Surviving Cells (10 ⁴)	Transformed Foci ^c	Frequency of Transformation (10 ⁻⁴) ^d	Mean Frequency of Transformation (10 ⁻⁴) $\pm$ 1 SE
Control ^b	(0.18)	2.10	0	0	0
TPA (0.16 μ M) ^b	0.93	3.74	0	0	0
Retinoid (7.1 μ M) ^b	0.75	1.58	0	0	0
Retinoid, TPA ^b	0.67	2.72	0	0	0
4 Gy	0.34	1.22	8	6.56	8.78 $\pm$ 1.29
	0.30	2.88	25	8.68	
	0.30	1.17	13	11.10	
4 Gy, TPA	0.31	1.53	16	10.45	16.15 $\pm$ 1.59
	0.58	3.23	60	18.58	
	0.68	1.39	27	19.42	
4 Gy, retinoid	0.26	1.14	6	5.27	4.37 $\pm$ 0.93
	0.32	2.46	10	4.07	
	0.33	1.58	6	3.79	
4 Gy, retinoid, TPA	0.36	2.89	16	5.54	2.46 $\pm$ 0.54
	0.39	2.70	5	1.85	
	0.32	1.14	0	0	

a Plating efficiency, (PE) = $\frac{\text{number of surviving colonies in control}}{\text{total cells plated}}$

Survival fraction, SF = $\frac{\text{number of surviving colonies}}{\text{total cells plated} \times (\text{PE})}$

b Values represent the average of three experiments

c Sum of types 2 and 3 transformations

d Frequency of transformation = $\frac{\text{transformed foci}}{\text{surviving cells}}$, Standard errors (SE) were determined assuming a Poisson distribution

Table 2. The Modulation of X-ray Induced Cell Transformation by TPA and All-Trans Retinoic Acid in Hamster Embryo Cells

Treatment	S.F.(PE) ^a	Total Colonies Counted	No. Colonies Transformed	Mean Frequency of Transformation ^c (10 ⁻³) $\pm$ 1 SE
Control	1.00	3800	0	0
TPA (0.16 μ M)	0.70	2612	0	0
^b Retinoid (7.1 μ M)	0.62	2424	0	0
Retinoid, TPA	0.89	2030	0	0
3 Gy	0.40	1064	8	6.99 $\pm$ 1.65
	0.44	1544	10	
3 Gy, TPA	0.48	1789	20	12.52 $\pm$ 1.81
	0.58	2019	28	
3 Gy, retinoid	0.31	1901	5	2.94 $\pm$ 0.79
	0.44	2768	9	
3 Gy, retinoid, TPA	0.41	1710	5	2.41 $\pm$ 0.85
	0.37	1590	3	

^aSurvival fraction (SF) = $\frac{\text{number surviving colonies}}{\text{total cells plated} \times \text{PE}}$

Plating efficiency (PE) = $\frac{\text{number of surviving colonies in control}}{\text{total cells plated}}$

^bAll-trans retinoic acid (β -RA)

^cStandard errors (SE) were determined assuming a Poisson distribution

TPA resulted in a substantial enhancement of the transformation frequency. In contrast, exposing cells to x-rays and retinoid reduced the transformation frequency by half. The combination of x-rays, continuous exposure to TPA and a 4 day exposure of cells to retinoid resulted in a transformation frequency similar to exposure of cells to x-rays and retinoid. Retinoid for only 4 days completely inhibited the promoting effects of a continuous exposure to TPA and much of the inducing effects of x-rays.

Sister chromatid exchanges

Results from 5 replicate experiments with the retinoid TMMP-ERA, and the one experiment with the retinoid TMMP-ERO are given in Table 3. The retinoid was in contact with the cells for the duration of the experiments, while TPA was added to the medium either before or after irradiation. One experiment involved irradiating cells *before* adding BrdU for 2 replication cycles (cells fixed 34 hours after irradiation) in the presence and absence of TPA. There were 0.55 and 0.57 SCE's per chromosome respectively (standard error of the mean 0.02 in each case). These figures contrast with 0.66 after 3 Gy alone and 0.83 after 3 Gy plus TPA at the time of irradiation (control incidence 0.47). This implies that while some damage initiated at the time of irradiation is later expressed as increased S.C.E.'s during the following replication cycles, many damaging events will not be detected by this procedure. In another series of additional treatments involving the retinoid TMMP-ERO, cells were irradiated with 3 Gy with retinoid present at the time of irradiation and throughout or added after irradiation. Cells were left for 28 hours then BrdU added and S.C.E.'s in second division metaphases examined 72 hours after irradiation. This protocol paralleled the transformation experiments where retinoid was also left on cells for 72 hours after irradiation.

Table 3. Mean Frequencies of Sister Chromatid Exchanges per Chromosome from Mouse C3H/10T $\frac{1}{2}$ Cells

Treatment	Experiments with retinoid TMMP-ERA					Retinoid TMMP-ERO
	1	2	3	4	5	
Control	0.31(0.01)	0.25(0.02)	0.23(0.02)	0.29(0.02)	0.47(0.02)	0.24(0.01)
TPA (0.16 μ M)	0.36(0.02)	0.24(0.02)	0.36(0.02)	0.26(0.02)	0.48(0.03)	- b
Retinoid (7.1 μ M)	0.35(0.02)	0.28(0.01)	0.34(0.02)	0.45(0.02)	0.44(0.02)	0.29(0.01)
TPA plus retinoid	0.27(0.01)	0.29(0.01)	0.25(0.02)	0.33(0.01)	0.47(0.02)	- b
3 Gy	0.58(0.03)	0.52(0.04)	0.47(0.02)	0.61(0.03)	0.66(0.03)	0.42(0.02)
3 Gy plus TPA	0.54(0.03)	0.62(0.03)	0.53(0.02)	0.53(0.02)	0.83(0.03)	- b
3 Gy plus retinoid	0.56(0.03)	- b	0.52(0.02)	0.76(0.02)	- b	0.33(0.02)
3 Gy plus TPA plus retinoid	0.67(0.03)	0.57(0.02)	0.64(0.03)	0.64(0.03)	0.83(0.03)	- b
3 Gy with TPA added after time of irradiation	- a	0.65(0.03)	0.53(0.02)	0.64(0.03)	- b	- b
Benzo(a)pyrene (2 μ M)	- a	1.23(0.04)	1.16(0.03)	1.17(0.04)	- a	- b

a No 2nd division cells present in the sampled population

b Mishandled or not done (TMMP-ERO)

c Standard error of the mean in brackets

The resulting S.C.E.'s per chromosome for all treatment groups were hardly different from control or retinoid alone indicating that the exchange frequencies can essentially return to control levels three divisions after irradiation and that potential retinoid effects do not include a later expression of damage. As is obvious from Table 3 there is some variability between experiments, but in general, both the TPA and the retinoid have little influence on sister chromatid exchange frequencies though both show small increases over the control. 3 Gy of x-rays, in general, increases SCE frequency by about 50% and both the TPA and the retinoid alone or in combination increase this level slightly. The levels of exchanges are, however, certainly enhanced when C3H/10T $\frac{1}{2}$ cells are incubated in the presence of BP for two cell cycles. With BP there is about a fivefold increase over control indicating that the cells were certainly capable of responding to an effective inducing agent.

In two of the five experiments there were only first division cells in the BP treated samples indicating that the movement of C3H/10T $\frac{1}{2}$ cells through their cell cycle is slowed considerably in the presence of this agent. In all other instances except one there were reasonable numbers of second division cells in the mitotic cell population with no indication that either TPA or retinoid alone or in combination influences the rate of progression of cells through their cycle. Certainly the 3 Gy dose of x-rays induced a mitotic delay however neither TPA nor retinoid further influenced cell progression. This is in agreement with observations on growth of these treated cells (Figures 2 & 4).

The dramatic contrast between the findings for sister chromatid exchange and for transformation induction in mouse C3H/10T $\frac{1}{2}$ cells is shown in Figure 5. Here the overall mean incidences of S.C.E. per chromosome are presented as a histogram for each treatment while the overall mean frequencies

Figure 5. Comparison of the effects of x-rays, retinoid TMMP-ERA and TPA on transformation induction (right side) and sister chromatid exchanges (left side) in C3H/10T $\frac{1}{2}$ cells. Standard errors of the mean are indicated. For the SCE studies cells were scored up to 20 hrs. after irradiation, while transformation incidence was assessed at 5 weeks after irradiation with TPA in continuous cell contact and retinoid present for 4 days only.

of transformations appear alongside. While TPA and retinoid slightly increase S.C.E. frequencies they produce no transformations. Radiation results in both increased S.C.E.'s and transformation, however, while addition of TPA or retinoid substantially enhances or reduces transformation, S.C.E.'s are comparatively unaffected. Further, while the added retinoid completely eliminates the promotional effect of TPA on radiation-induced transformation, S.C.E.'s are slightly increased and it appears as if the increase over radiation alone is due to the small additive effects of TPA and retinoid.

Discussion

These results address two primary areas of concern when examining the interaction of retinoids and the promoter TPA on radiation induced oncogenic transformation. First, mouse C3H/10T $\frac{1}{2}$ cells and Syrian hamster embryo cells show that not only can retinoids reduce the frequency of *in vitro* transformation induced by ionizing radiation, but they can completely eliminate the substantial promotional effects of TPA. The ability of retinoids to inhibit the promotional effects of TPA occurs even when cells are in chronic exposure to TPA and only exposed to retinoids for 4 days.

Merriman and Bertram (15) found that the inhibition of methycholanthrene induced transformation by all trans retinyl acetate was essentially completely reversible when retinoid treated C3H/10T $\frac{1}{2}$ cells were later grown for 4 to 5 weeks in drug-free medium. In this study, when radiation was the transformation inducer and retinoid treatment time was confined to 3 days post irradiation (for both cell systems), a profound inhibitory effect was definitely sustained.

Explanation of the antagonistic effects between retinoids and TPA is made more difficult by two further observations. First the high cell density seen in the photomicrograph in Figure 1 and the final cell number in Figure 4 for cells

exposed to TPA were unchanged with the addition of retinoid. Second, although transformed cells had a growth rate less than normal cells when both cell types were grown in retinoid, this difference is hardly adequate to account for the inhibition of x-ray induced transformation by retinoids.

This finding leads to the second area of concern dealing with the manner of retinoid and TPA influence on radiation induced transformation. Sister chromatid exchanges are indicators of damage at the level of DNA. Thus, the frequencies of S.C.E.'s provide a basis for evaluating whether the effects of TPA and retinoids are at the initiation or expression stages of carcinogenesis. Our results suggest that these compounds have little or no influence on the initiation process. This then implies that the expression phase is the more susceptible to these agents. An enhancing effect of retinoids on S.C.E. induction has been claimed to have substantial effects on exchange induction (11). The substantially negative finding with TPA in this study is in agreement with the later studies of Loveday and Latt (14) and Thompson et al. (22) and mitigates against the proposal of Kinsella and Radman (10) that TPA influences transformation by increasing somatic recombination. The failure of the S.C.E.'s to follow the effect of either agent on transformation incidence also implies that while S.C.E.'s may be useful as indicators of primary carcinogen-mutagens (17) they have little utility when secondary agents alter the response of cells to a primary initiator.

The retinoids TMMP-ERA and β -RA are powerful antagonists of the promoting effect of TPA on radiation induced transformations *in vitro*. Although the mechanisms for the interaction of these compounds is yet to be fully understood, reduction of induced transformation by retinoids suggests a promising future in clinical application.

References

1. Alyes, P., and Johnasson, J. New staining method for the detection of sister chromatid exchanges in BUdR labelled chromosomes. *J. Cell Sci.*, 32:185-195 (1978).
2. Bollag, W. Therapy of chemically induced skin tumors of mice with vitamin A palmitate and vitamin A acid. *Experientia*, 27:90-92 (1971).
3. Borek, C. Malignant transformation *in vitro*: Criteria, biological markers, and application in environmental screening of carcinogens. *Radiat. Res.*, 79:209-232 (1979).
4. Borek, C., and Sachs, L. *In vitro* transformation by x-irradiation. *Nature*, 210:276-278 (1966).
5. Goodman, DeW. S. Vitamin A and retinoids: recent advances. Introduction, background, and general overview. Symposium presented by the Am. Inst. of Nutrition at the 63rd Annual Meeting of the Fed. of Am. Soc. for Experimental Biol. Dallas, April, 1979.
6. Harisiadis, L., Miller, R.C., Hall, E.J., and Borek, C. A vitamin A analogue inhibits radiation-induced oncogenic transformation. *Nature*, 274:486-487 (1978).
7. Juhl, H.J., Schurer, C.C., Bartram, C.R., Kohl, F.V., Melderis, H., Wichert, P.V., and Rudiger, H.W. RETinoids induce sister-chromatid exchanges in human diploid fibroblasts. *Mutation Res.*, 58:317-320 (1978).
8. Kennedy, A.R., Mondal, S., Heidelberger, C., and Little, J.B. Enhancement of x-ray transformation by 12-o-tetradecanoyl-phorbol-13-acetate in a cloned line of C3H mouse embryo cells. *Cancer Res.*, 38:439-443 (1978).

9. Kensler, T.W., and Mueller, G.C. REtinoic acid inhibition of the comi-
togenic action of mezerin and phorbol ester in bovine lymphocytes.
Cancer Res., 38:771-775 (1978).
10. Kinsella, A.R., and Radman, M. Tumor promoter induces sister chromatid
exchanges: Relevance to mechanisms of carcinogenesis. Proc. Natl. Acad.
Sci. (USA), 75:6149-4153 (1978).
11. Little, J.B., Nagasawa, H., and Kennedy, A.R. DNA repair and malignant
transformation: effect of x irradiation, 12-o-tetradecanoyl-phorbol-13-
acetate, and protease inhibitors on transformation and sister-chromatid
exchanges in mouse 10T $\frac{1}{2}$ cells. Radiat. Res., 79:241-255 (1979).
12. Lotan, R. Effects of vitamin A and its analogs (retinoids) on normal
and neoplastic cells. Biochem. Biophys. Acta., Reviews on Cancer 1980
(In press).
13. Lotan, R. Retinoic acid stimulates and phorbol myristate acetate inhibits
melanogenesis in cultures of murine S91 melanoma cells. J. Cell Biol.,
79:CD 144 (Abstracts) (1978).
14. Loveday, K.S., and Latt, S.A. The effect of a tumor promoter, 12-o-
tetradecanoyl-phorbol-13-acetate (TPA) on sister-chromatid exchange
formation in cultured Chinese hamster cells. Mutation Res., 67:
343-348 (1979).
15. Merriman, R.L., and Bertram, G.S. Reversible inhibition by retinoids
of 3-methylcholanthrene-induced neoplastic transformation in
C3H/10T $\frac{1}{2}$ /clone 8 cells. Cancer Res., 39:1661-1666 (1979).
16. O'Brien, T.G., and Diamond, L. Metabolism of tritium labeled
tetradecanoyl phorbol-13 acetate by cells in culture. Cancer Res.,
38:2562-2566 (1978).

17. Perry, P., and Evans, H.J. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. *Nature (London)*, 258:121-125 (1975).
18. Peterson, A.R., Mondal, S., Brankow, D.W., Thon, W., and Heidelberger, C. Effects of promoters on DNA synthesis in C3H/10T $\frac{1}{2}$ mouse fibroblasts. *Cancer Res.*, 37:3223-3227 (1977).
19. Reznikoff, C.A., Bertram, J.S., Brankow, D.W., and Heidelberger, C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.*, 33:3239-3249 (1973).
20. Reznikoff, C.A., Brankow, D.W., and Heidelberger, C. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.*, 33:3231-3238 (1973).
21. Sporn, M.B., and Newton, D.L. Chemoprevention of cancer with retinoids. *Fed. Proc.*, 38:2528-2534 (1979).
22. Thompson, L.H., Baker, R.M., Carrano, A.V., and Brookman, K.W. Failure of the phorbol ester TPA to enhance sister chromatid exchange, mitotic segregation or expression of mutations in Chinese hamster cells. *Cancer Res.* (In press).
23. Todaro, G.J., De Larco, J.E., and Sporn, M.B. Retinoids block phenotypic cell transformation produced by sarcoma growth factor. *Nature (London)*, 276:272-274 (1978).
24. Verma, A.K., and Boutwell, R.K. Vitamin A acid (retinoic acid), a potent inhibitor of 12-o-tetradecanoyl-phorbol-13-acetate-induced ornithine decarboxylase activity in mouse epidermis. *Cancer Res.*, 37:2196-2201 (1977).

25. Weeks, C.E., Slaga, T.J., Hennings, H., Gleason, G.L., and Bracken, W.M. Inhibition of phorbol ester-induced tumor promotion in mice by vitamin A analog and anti-inflammatory steroid. JNCI, 63:401-406 (1979).
26. Weinstein, I.B., Yamasaki, H., Wigler, M., Lee, L.S., Fisher, P.B., Jeffrey, A., and Grunberger, D. Molecular and cellular events associated with the action of initiating carcinogens and tumor promoters. Carcinogens: Identification and Mechanisms of Action, edited by A. Clark Griffin and Charles R. Shaw, Raven Press, New York, 399-418 (1979).

RB-4. X-ray-induced *in vitro* neoplastic transformation of human diploid cells.

Carmia Borek

Techniques have recently been developed to identify and score quantitatively neoplastic transformation caused by x-rays in cultured cells derived from rodents¹. Because of their relevance to estimates of human cancer risk, it is clearly desirable to carry out similar experiments with cultured cells of human origin. The present report describes for the first time the neoplastic transformation *in vitro* of human diploid cells by x-ray irradiation into cells which can progress *in vitro* into advanced stages of neoplastic development, namely, to form colonies in agar and give rise to tumours when injected into nude mice.

We used an early passage of skin fibroblast strain KD, cells, originally derived from a skin biopsy of an adult woman^{2,3}. The cultures were in their fifth cell passage, dating from the original biopsy. Chromosome G banding analysis carried out on the cells on arrival indicated a normal diploid human karyotype. The doubling time of the KD cells as determined by growth curve analysis⁴ was 30-32 h.

The cells were routinely maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum and 1% human serum (GIBCO) and incubated at 37°C. A dose-response curve for cell survival was carried out as described elsewhere⁵ (see Figure RB-4-1). The scheme for transformation experiments is described in detail in Figure RB-4-2 legend. All cells were fed twice a week. Four separate experiments have been carried out using antipain⁶ or β -oestradiol. In each experiment control cultures included unirradiated, untreated cells as well as cells unirradiated, but treated with antipain or β -oestradiol. These were set up in equal numbers to the experimental flasks and subcultured in the

Figure 1: Cell survival of KD cells following irradiation by X-rays from a Sieman's 300 kVp constant potential generator with an added filter of 0.2 mm copper.

same manner.

Within 2 months of irradiation, discrete foci were detected in the pre-treated, irradiated cultures which had been left untouched following the 1:12 split and were now at high density. The foci, consisting of cells with a subtle irregularity in cell to cell orientation, were visible under phase microscopy. Two or three foci were initially detected in each experiment in both antipain- and oestradiol-pretreated, irradiated cultures, suggesting that these compounds were equally effective in potentiating radiation-induced transformation. Within 3 months, 30-40 of these foci per experiment were observed in most of the high-density flasks but not in those which had been given a further subculture (Figure RB-4-2). The latter exhibited these foci after transferring the cells at high density (2×10^6) to 100-mm Petri dishes.

The foci increased in size. A multilayering of cells made them clearly visible to the naked eye (Figure RB-4-3). No foci appeared in the unirradiated cells or in cells treated with antipain or oestradiol alone.

By exchanging the medium for medium containing low calcium concentrations⁷, the cells in the morphologically transformed foci underwent an additional, more dramatic morphological alteration (Figure RB-4-3), whereas the normal cells degenerated within 24 h.

Chromosomal G banding analysis carried out on cells isolated from the primary foci showed a human chromosomal constitution with a diploid to near-diploid number of chromosomes (range 46-49). A more detailed karyotype will be published elsewhere. Saturation density determined on the transformed cells cultured from the foci showed a twofold increase in saturation density over that of the normal. The transformed cells but not the normal parental cells were agglutinable by $25 \mu\text{g ml}^{-1}$ of concanavalin A (Miles-Yeda).

Figure 2:

Experimental design for the induction of human cell transformation in vitro by 4 gray of X-rays (1 gray = 100 rad). Cells were trypsinised (0.25% trypsin), suspended in complete medium and plated in tissue culture flasks (75 cm²) (Falcon) at 2.6×10^5 cells. Two days later, the medium was replaced with MEM containing 0.1% serum for 24h, whereby cell proliferation was greatly reduced. After 24h, medium was again exchanged, this time for complete medium (11% serum) containing either $6 \mu\text{g ml}^{-1}$ of the protease inhibitor antipain⁶ or $1 \mu\text{g ml}^{-1}$ β -oestradiol (Sigma). Experimental cultures were irradiated 12 h later with 4 gray of x-rays as previously described⁵. All cultures were divided into two 10-12 h after irradiation and reseeded in fresh complete MEM without antipain or oestradiol. At near confluency, one of the two flasks was subcultured into 12 flasks, the other being left undisturbed. In each experiment, when these 12 flasks reached confluency, one flask was subcultured 1:10 and thus defined as "continuously passaged", and the remaining 11 flasks were left at high density.

Figure 3:

- a, Human KD cells in irradiated but untransformed cultures. Phase x58.
- b, Human KD cells transformed in vitro by 4 gray. Note the criss-cross pattern of the cells. Phase x58.
- c, The same area as in b 24 h after incubation in low calcium. Phase x58.
- d, X-ray-transformed KD cells growing in 0.33% agar. x12.

The ability to proliferate at low serum concentrations (1%) was not confined to the transformed human cells⁸ as in rodent systems^{1,4}, but was also a property of the normal KD cells grown in the same conditions (10^5 cells per 60-mm Petri dish).

Cells isolated from the multilayered foci were seeded in semi-solid agar⁹, where they proliferated to form distinct spherical colonies (Figure RB-4-3) with a cloning efficiency of 2-3%. None of the unirradiated KD cells or the irradiated but untransformed cells (seven samples of each) was able to proliferate in agar. Colonies isolated from agar could be recultured in liquid medium or further propagated in agar.

The ultimate proof of the neoplastic nature of the cells transformed *in vitro* is their ability to form tumours in an appropriate host. Swiss nu/nu mice were injected intradermally with 5×10^6 cells (suspended in 1 ml medium) into the subscapular region of animals x-ray-irradiated 24 h earlier with 450 rad or unirradiated¹⁰.

Whereas the five transformed lines tested formed colonies in agar, three gave rise within 6 weeks both in the irradiated and unirradiated mice to tumours which have been characterized as noninvasive fibrosarcomas. Cells cultured from the tumours possessed a human karyotype which will be described in detail elsewhere. None of the unirradiated or the irradiated but untransformed cells (seven samples of each) gave rise to tumours in the nude mice.

The present experiments show that human diploid cells can be transformed *in vitro* into tumorigenic cells by x-ray-irradiation. Cultures which were treated and irradiated in the non-dividing state, as well as those which were allowed only four or five cell divisions before reaching confluency (and not subcultured further), did not exhibit transformed foci. This indicates that cell replication

is required for the fixation and expression of radiation-induced neoplastic transformation in diploid human cells, and a quantitative assessment is now in progress. This requirement has been shown to exist in the case of rodent cells transformed *in vitro* by radiation¹¹⁻¹³ or chemicals^{14,15} and in human cells transformed *in vitro* by chemicals³. It was of interest that cells which were not recognisable as transformed at low density expressed transformation when replated at high density in close contact.

The complex experimental design used here takes advantage of the following. (1) The cells which were well adapted to complete medium were subjected to conditions of serum deprivation for a period of 24 h, thus reaching a complete quiescence, indicated in these conditions by specific conformational changes in chromatin (Hoen, personal communication). (2) The addition of complete medium 24 h later led to a synchronous wave of DNA synthesis and treatment of the cells at that point enabled the capturing of cells entering S phase.

We do not know the mechanism of action of oestradiol and antipain in this system. Neither compound exhibited mitogenic activity at the concentration used and both seemed equally effective. We have previously shown that antipain, a protease inhibitor, when present in cultures during irradiation enhanced the yield of x-ray-induced neoplastic transformation in hamster and mouse cells¹⁶. Oestradiol has been used to potentiate chemically induced transformation¹⁷ and although steroids have many functions, they do have some protease inhibitor activity¹⁸. Thus, one could speculate that the enhancing action of both compounds may be in part through a common mechanism related to this property.

Although the above experimental regime has been successful for the induction and detection of radiation-induced transformation in the KD cells, it must be stressed that we are relatively ignorant of the best experimental design for

inducing transformation and detecting transformants in human cells. Note, for example, that although partial cell synchrony and potentiation of transformation by hormones was a necessary step in the transformation of some diploid human cells by chemical carcinogens¹⁷, it was not required for the induction of transformation in other diploid cells³. Because the latter were, in fact, the KD cells, we are now evaluating other experimental designs to induce and detect transformation by radiation in the human cells. The ability to transform human cells *in vitro* by x-ray irradiation opens up a wide range of studies which should aid in the assessment of human risk to radiation-induced carcinogenesis and in the evaluation of treatments to inhibit its expression and progression.

References

1. Borek, C., Radiat. Res. 79, 209-232 (1979).
2. Day, R.S., Cancer Res. 34, 1965-1970 (1975).
3. Kakunaga, T., Proc. Natn. Acad. Sci., U.S.A. 75, 1334-1338 (1978).
4. Borek, C., Pain, C. and Mason, H., Nature 266, 452-454 (1977).
5. Borek, C. and Hall, E.J. Nature 243, 450-453 (1973).
6. Umezawa, H., Meth. Enzym. 45, 678-695 (1977).
7. Boynton, Z.L. and Whitfield, J.F. Cancer Res. 38, 1237-1240 (1978).
8. Borek, C., Abstr. 6th Int. Cong. Radiat. Res., Tokyo (1979).
9. Borek, C., Proc. Natn. Acad. Sci. U.S.A. 69, 956-959 (1978).
10. Borenfreund, E., Higgins, P.J., Steinglass, M. and Bendich, A., J. Natn. Cancer Inst. 55, 375-384 (1975).
11. Borek, C. and Sachs, L., Proc. Natn. Acad. Sci. U.S.A., 57, 1522-1527 (1967).
12. Borek, C. and Sachs, L., Proc. Natn. Acad. Sci. U.S.A. 59, 83-85 (1968).
13. Terzaghi, M. and Little, J.B., Cancer Res. 36, 1367-1374 (1976).

14. Chen, T.T. and Heidelberger, C., *Int. J. Cancer* 4, 166-178 (1969).
15. Kakunaga, T., *Cancer Res.* 35, 1637-1642 (1975).
16. Borek, C., Miller, R., Pain, C. and Troll, W., *Proc. Natn. Acad. Sci. U.S.A.* 76:1800-1803 (1979).
17. Milo, G.E. and DiPaolo, J.A., *Nature* 275, 130-132 (1978).
18. Troll, W., in *Fundamentals in Cancer Prevention* (eds Magee, P.N. et al.) 41-50 (University of Tokyo Press, Baltimore, 1976).

RB-5 Liver Cells in Culture: A Model for Investigating the Regulation of Retinol-Binding Protein Metabolism.

Carmia Borek in collaboration with John Edgar Smith and DeWitt S. Goodman (Department of Medicine)

In recent years it has become increasingly clear that retinoids have multiple functions. Vitamin A and its analogs influence a variety of parameters in cell growth and differentiation (for reviews see 1-3) and in addition are able to modulate the expression of the malignant state and inhibit neoplastic development *in vivo* and *in vitro* (for reviews see 4, 5). While the activity of retinoids as inhibitors of neoplastic transformation *in vitro* has been pursued (6) the present discussion will be devoted to their action *in vitro* on differentiation and will concentrate on the regulation of the retinol binding protein (7) by vitamin A.

Retinol, a water soluble lipid alcohol is the major form of vitamin A normally found in the plasma. Retinol circulates in the plasma bound to retinol binding protein (RBP) (7) which has been purified from a variety of species including humans (7) and rats (8,9). RBP is a small protein with a molecular weight of about 20,000 daltons, which has a single binding site for one molecule of retinol. In plasma holo-RBP circulates as a protein-protein complex with prealbumin (10). RBP is synthesized in the liver and secreted mainly as the holo-protein (with bound retinol) (11, 12). After the delivery of retinol to vitamin A requiring tissues, the resulting apo-RBP has a lower affinity for prealbumin (13). Apo-RBP is rapidly cleared from the circulation by glomerular filtration in the kidney (14).

The secretion of RBP from the liver is regulated in a specific manner by

the availability of retinol (11, 12, 15). Thus, vitamin A deficiency results in a block in RBP secretion from the liver and an accumulation of apo-RBP in the hepatic cells. After supplementation of the diet with retinol the accumulated RBP is released rapidly into the plasma as holo-RBP (12).

Information on the secretion of RBP and its regulation has been obtained heretofore from *in vivo* studies. The complexity of the homeostatic mechanisms *in vivo* present limitations when trying to evaluate the interplay among various factors which may modulate RBP production and secretion. For this reason and for the purpose of investigating cellular and molecular mechanisms of RBP production and secretion, it seemed desirable to find an *in vitro* model of liver cells in culture which would produce RBP and be responsive to a variety of factors which regulate RBP *in vivo*.

RBP producing cell lines

Two cell lines derived from differentiated hepatomas (16) were found to be suitable models; the MH_1C_1 cell line (17) and the $H_4II EC_3$ (18). While normal hepatocytes in culture tend to lose some of their differentiated function in long term culture (19, 20) the neoplastic differentiated cells maintained their differentiated properties over a span of many years (21).

The experimental results derived from using the MH_1C_1 line and the $H_4II EC_3$ (H_4) were qualitatively similar (22). However, because of the fact that the MH_1C_1 had a doubling time of 5 days in contrast to the 24 hrs of the H_4 cells, the H_4 cell line ultimately became the model of choice (22).

The H_4 line

Originally derived and cloned from an H-35 tumor (18) the H_4 hepatoma cells grow in monolayers of polygonal cells (Figs. 1 & 2) which at high cell densities tend to pile up and form multilayers (23). The topography of the

Fig. 1 Two H_4 II EC_3 (H_3) cells in culture. Note the microvilli on the cell surface and the intercellular bridges. SEM x600.

Fig. 2 H_4 cells culture an interphase cell and a cell in mitosis. Note the abundant surface microvilli on the mitotic cell. SEM x600.

cells as seen by scanning electron microscopy is characterized by multiple microvilli on the cell surface, both in interphase and especially in mitosis (Fig. 2). Cell-cell junctions are characterized by intercellular bridges (Fig. 1), though a lack of ionic communication at permeable intercellar membrane junctions has been observed (24). The tumorigenicity of the cells, the lack of intercellular communication and an ability of the cells to be agglutinated by various plant lectins (25) differentiate them from normal hepatocytes (24,25). In addition, the karyotype analysis of the H₄ cells (Fig. 3) using banding methods (26) differs from normal. H₄ cells are heteroploid (26). There are about 50 chromosomes/cell in the H₄ line, 80% of which are normal. Though each cell had about 40 normal chromosomes their distribution was such that none of the cells had a diploid complement of chromosomes, and none of the 20 cells examined was exactly like any other. Each H₄ hepatoma cell contains an average of 9 abnormal marker chromosomes. In view of these marked deviations from normal karyotype it is indeed amazing that the H₄ cells have retained many differentiated properties, including the ability to synthesize RBP, albumin and prealbumin (22), in a reproducible manner over the years. The H₄ cells used in our experiments were obtained from Dr. Van Potter and were maintained in F12 medium supplemented with 10% fetal calf serum (22). RBP and albumin were assayed by radioimmuno assays (22), while total cellular protein was assayed by the Lowry method (22). All culture techniques and biochemical procedures used in the experiments have been described in detail (22).

Medium of choice

In order to effectively establish a cell model for the study of RBP production and its regulation by retinol it was essential to find a growth medium in which no retinol would be present. Normal serum contains trace amounts of retinol which may vary from one serum batch to another. Thus a

Fig. 3 Karyotype of the H_4 cells. Note the chromosome markers. Reproduced with permission from (26)

serum free medium seemed an optimal solution. The Neuman and Tytell serumless medium (NTS) (GIBCO) was found to be suitable. In it, the H_4 cells could be maintained as a stationary culture in G_0 (no thymidine uptake was observed upon pulse label (22)). The cells in the serumless medium were in semi-suspension. In order to evaluate whether the physical state of the H_4 cells (in suspension vs. monolayer) would modulate RBP production in the cells or its secretion from the cells, glutathione was added to the medium at 15 $\mu\text{g/ml}$ causing most of the cells to adhere to the dish. There was no difference in RBP production or secretion in the cells with glutathione added to the NTS when compared to the cells in semi-suspension maintained in NTS alone (22).

Optimal cell density and incubation time

Once it was ascertained that the hepatoma cells synthesized and secreted RBP as assayed in cellular extracts and in the medium the following parameters were studied in order to characterize the cell system:

- a) The effect of cell density on RBP production.
- b) The effect of incubation time in culture on the production of RBP.

The results of the study are described in detail elsewhere (22) for both H_4 cells and $MH_1 C_1$ cells (22).

Net synthesis of RBP, defined here as an increase in total RBP (cells and medium) observed during the time of incubation, was proportional to the length of incubation. The net synthesis of RBP was directly proportional to the number of cells seeded initially in the Petri dishes.

Effects of retinol

The hepatoma cells in culture maintained in the NTS medium which contains no vitamin A represent a system of vitamin A deficient liver cells (22). In the

NTS medium cells retain from 14-56% of the total RBP within the cell and thus resemble the liver of a vitamin A deficient rat.

The response of the H₄ cells to retinol was investigated by adding 0.35 or 3.5 nmol retinol/ml to the NTS medium 24 hrs after seeding the cells. As seen in Fig. 4, retinol clearly stimulated RBP secretion into the medium within one day and led to a small increase in total RBP. The decrease in level of RBP in the cells was particularly evident when expressed as pmole RBP per mg cell protein. This cellular depletion presumably reflected increased secretion of RBP into the medium. The effect of retinol was specifically directed at RBP as seen by the fact that there was no change in the total level of albumin nor its distribution between the cells and the medium, whether retinol was present for one day (not shown) or 3 days (Fig. 4). The lack of effect on albumin also indicated that the retinol treated cells had not undergone cellular damage and that the cellular response to retinol in modifying RBP production was a true effect directly influenced by retinol.

Other hormonal effects on RBP

Recently we have been investigating the regulation of RBP synthesis by glucocorticoid hormones, in particular using the agent dexamethasone. The questions asked were:

- a) Does dexamethasone have an effect on RBP synthesis?
- b) If it does, is the effect additive or synergistic with that of retinol?

The results so far in this ongoing work indicate the following:

- a) Dexamethasone at concentrations of 10^{-7} M enhances the synthesis and secretion of RBP.
- b) The dexamethasone enhancing effect is synergistic with the retinol effect, so that 10^{-8} dexamethasone plus retinol elicits a seven

Fig. 4 Effect of retinol on the net synthesis and secretion of RBP from H_4 cells. The bars represent the mean of two values. The individual values are shown as dots. The black area at the bottom of each bar represents the cellular RBP or albumin, while the open or cross-hatched areas of the bars represent the RBP or albumin in the medium, H_4 cells were incubated in 60 mm petri dishes in 5 ml of NTS medium with 6×10^6 cells per dish for either 1 or 3 days. Retinol was added to the medium of the designated dishes at the beginning of the incubation. Two concentrations of retinol were studied: 0.35 and 3.5 nmole/ml (corresponding to 0.1 and 1.0 $\mu\text{g/ml}$ respectively). For statistical comparisons, data from the two control (no added retinol) treatments were pooled and the data from the two retinol treatments were pooled. In both the 1 day and 3 day incubations, the level of RBP in the cells was significantly reduced by the addition of retinol ($p < 0.005$), and the level of RBP in the medium was significantly increased by the addition of retinol ($p < 0.005$, 1 day; $p < 0.01$, 3 days). Moreover, the addition of retinol increased the total RBP (cell + medium) with both 1 day ($p < 0.05$) and 3 day ($p < 0.025$) incubation, are shown in the right-hand panel. The treatment designated "0 retinol added" had no additions; the treatment designated "0" had 50 μl of the retinol carrier solution added. Reproduced with permission from (22).

fold increase in RBP production, a response greater than the one obtained following incubation with 10^{-7} dexamethasone alone.

- c) The enhancing effect of dexamethasone is inhibited by progesterone a hormone which is a competitive inhibitor for the steroid receptor site in the action of dexamethasone. This inhibitory effect is specific since albumin and prealbumin are unaffected.

These findings, which will be reported in detail elsewhere, strongly suggest that the enhancing effect of dexamethasone on RBP production is mediated by specific steroid hormone receptors and not by a stabilization of m-RNA. Additional support for a receptor mechanism is suggested by the fact that the addition of dexamethasone at concentrations as low as 10^{-9} M result in a significant increase in the net synthesis of RBP. This type of enhancement would not be expected had dexamethasone been acting as a stabilizer.

From the results described here and elsewhere (22) it appears that the H_4 cell line as well as the $MH_1 C_1$ (22) are effective model systems for studying the regulation of RBP metabolism in the liver cell. Not only do these cells produce RBP in culture, but they also respond to the addition of retinol in a manner similar to that of the liver cell in the intact liver *in vivo*. The ability of the cells to respond to other hormones, which may modulate RBP metabolism but not that of other plasma proteins, enhances their value as an RBP producing cell model.

Further work will help to elucidate both cellular and molecular mechanisms underlying the production and secretion of RBP and its regulation.

References

1. Wolback, S.B. and Howe, P.R. Tissue changes following deprivation of fat soluble Vitamin A. J. Exp. Med. 43:753-777 (1925).
2. Goodman, DeW. S. Vitamin A and retinoids: Recent advances. Fed. Proc. 38: 2501-2503 (1979).
3. Chytil, F. and Ong, D.E. Cellular retinol and retinoic acid binding protein in Vitamin A action. Fed. Proc. 38:2510-2514 (1979).
4. Sporn, M.B. and Newton, D.L. Chemoprevention of cancer with retinoids. Fed. Proc. 38:2528-2534 (1979).
5. Lotan, R. Effects of Vitamin A and its analogs (retinoids) on normal and neoplastic cells. Bioch. Bioph. Acta. In Press.
6. Harisiadis, L., Miller, R.C., Hall, E.J. and Borek, C. A Vitamin A analogue inhibits radiation-induced oncogenic transformation. Nature (London) 274:486-487 (1978).
7. Kanai, M., Raz, A., and Goodman, DeW.S. Retinol-binding protein: the transport protein for Vitamin A in human plasma. J. Clin. Invest. 47: 2025-2044 (1968).
8. Peterson, P.A., Rask, L., Ostberg, L., Andersson, L., Kamwendo, F. and Pertoft, H. Studies on the transport and cellular distribution of Vitamin A in normal and Vitamin A-deficient rats with special reference to the Vitamin A-binding plasma protein. J. Biol. Chem. 248:4009-4022 (1973).
9. Muto, Y. and Goodman, DeW.S. Vitamin A transport in rat plasma, isolation and characterization of retinol-binding protein. J. Biol. Chem. 247:2533-2541 (1972).

10. Goodman, DeW.S. Retinol-binding protein, prealbumin and Vitamin A transport in trace components of plasma, isolation and clinical significance. G.A. Mamieson and T.J. Greenwait, eds. (New York: Alan R. Liss), pp. 313-330 (1976).
11. Muto, Y., Smith, J.E., Milch, P.O. and Goodman, DeW. S. Regulation of retinol-binding protein metabolism by Vitamin A status in the rat. J. Biol. Chem. 247:2542-2550 (1972).
12. Smith, J.E., Muto, Y., Milch, P.O. and Goodman, DeW.S. The effects of chylomicron Vitamin A on the metabolism of retinol-binding protein in the rat. J. Biol. Chem. 248:1544-1549 (1973).
13. Smith, F.R. and Goodman, DeW.S. The effects of diseases of the liver, thyroid and kidneys on the transport of Vitamin A in human plasma. J. Clin. Invest. 50:2426-2436 (1971).
14. Rask, L. and Peterson, P.A. Structure and function of the retinol binding protein: A protein characteristic of tubular proteinuria. Protides Biol. Fluids 21:485-491 (1974).
15. Navab, M., Smith, J.E. and Goodman, DeW.S. Rat plasma prealbumin: metabolic studies on effects of Vitamin A status and on tissue distribution. J. Biol. Chem. 252:5107-5114 (1977).
16. Morris Hepatomas. H. Morris and W.E. Criss (eds.) Plenum Press, N.Y. (1978).
17. Richardson, U.I., Tashjian, A.J., Jr. and Levine, L. Establishment of a clonal strain of hepatoma cells which secrete albumin. J. Cell Biol. 40:236-247 (1969).
18. Pitot, H.C., Peraino, C., Morse, P.A. Jr. and Potter, V.R. Hepatomas in tissue culture compared with adapting liver *in vivo*. Nat. Cancer Inst. Monograph 13:229-245 (1964).

19. Borek, C. Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated hepatoma like cells under conditions of nutritional stress. Proc. Natl. Acad. Sci. U.S. 69:956-959 (1972).
20. Borek, C. Differentiation, metabolic activation and malignant transformation in cultured liver cells exposed to chemical carcinogens. In: Mammalian Cell Transformation by Chemical Carcinogens, V. Kunkel, N. Mishra and M. Mehlman (eds.) In press.
21. Gene Expression and Carcinogenesis in Cultured Liver. L. Gerschensohn and G.B. Thompson (eds.) Academic Press, New York (1975).
22. Smith, J.E., Borek, C. and Goodman, DeW. S. Regulation of retinol-binding protein metabolism in cultured rat liver cell lines. Cell, Vol. 15, 865-873 (1978).
23. Borek, C. Surface membranes and biological regulation in differentiated hepatoma cells *in vitro*. In: Morris Hepatomas, H.P. Morris and W.E. Criss (eds.) Plenum Press, New York (1978).
24. Borek, C. Studies on normal and neoplastic liver cells in culture contact behavior, cellular communication and transformation. In: Gene Expression and Carcinogenesis in Cultured Liver. L. Gerschensohn and G.B. Thompson (eds.) Academic Press, New York 1975.
25. Borek, C., Grob, M. and Burger, M.M. Surface alterations in transformed epithelial and fibroblastic cells in culture: A disturbance of membrane degradation versus biosynthesis? Exp. Cell Res. 77:207-215 (1973).
26. Miller, D.A., Dev, V.G., Borek, C. and Miller, O.J. The quinacrine fluorescent and giemsa banding karyotype of the rat, *Rattus norvegicus*, and banded chromosome analysis of transformed and malignant rat liver cell lines. Cancer Res. 32:2375-2382 (1972).

Eric J. Hall and Myles Astor

Neutron therapy is actively underway at a number of centers in Great Britain, the United States, Continental Europe and Japan, using a wide range of neutron energies. Most Clinical facilities utilize the $d^+ \rightarrow Be$ or $p^+ \rightarrow Be$ reactions, with the energy of the accelerated particle ranging from 16 MeV at Hammersmith to 67 MeV at the Fermilab. Over this range of neutron energies, the Oxygen Enhancement Ratio (OER) appears to be relatively constant at 1.5 to 1.6, with the possibility of a small gradual increase in OER with neutron energy (Hall and Kellerer, 1979; Hall et al., 1977). The reduced oxygen effect is the principal rationale for the introduction of neutrons into clinical practice as an alternative to x or γ -rays, so the value of the OER is of basic importance. There have been reports in the literature that neutron energies higher than those currently in use may result in a lower OER; reports of experiments at the University of Maryland cyclotron indicated OER values of 1.3 and 1.2 for neutrons generated by 80 MeV deuterons and 101 MeV protons on beryllium respectively (Harrison et al., 1975, 1976). This possibility is obviously of great interest and importance from the point of view of choosing the energy range for future clinical facilities since a higher neutron energy would accrue the additional benefits of improved depth doses and a higher dose-rate.

Because of the practical importance of this subject we attempted in 1977 to repeat the work of Harrison and colleagues at the University of Maryland, making measurements with the 80 MeV $d^+ \rightarrow Be$ beam. However, we found the OER to be high at about 1.7 (Hall et al., 1977) using either hamster cells *in vitro* or *via* seedlings, consistent with our previous report for the neutrons generated at the

Fermilab by 67 MeV $p^+ \rightarrow Be$. At that time, the 101 MeV proton beam could not be used because of a major cyclotron breakdown. The high energy proton beam has recently become available again and the present communication reports the results of experiments with this beam and also with a beam generated by 80 MeV deuterons on a thin combination target of beryllium and tantalum, which results in a neutron spectrum with a higher average energy than the thick target used in our previous experiments at 80 MeV. The dosimetry was kindly performed by Dr. George Harrison.

Cells were irradiated while sealed in glass ampoules which were vigorously shaken during irradiation to keep the cells in suspension and prevent them from settling out on the glass surface. Tissue equivalent plastic, 4 cm thick, was used to achieve build-up of dose. Hypoxia was produced by using a high cell concentration (2×10^6 cells in 1 ml of medium), flushing air from the ampoule with a mixture of 5% CO_2 in nitrogen, and then incubating the sealed ampoule for one hour at $37.5^\circ C$ so that residual oxygen was scavenged from the system by cell respiration. A second series of ampoules contained 10^4 cells and remained aerated. After irradiation, the ampoules were opened and aliquots of the cell suspension replated to test for colony formation. This technique has been described in detail elsewhere (Hall et al., 1974, 1975). Survival curves under aerated and hypoxic conditions are shown in Figures RB-6-1 and RB-6-2 for the neutron beams generated by 80 MeV $d^+ \rightarrow Be$ and 101 $p^+ \rightarrow Be$; the OER values obtained were about 2.1 and 1.9 respectively. These should be compared with an OER value for x-rays measured by this technique of about 3.0 to 3.2.

The results of this series of experiments are in direct conflict with the published data of Harrison and his colleagues. We see no indication of a reduced OER for higher neutron energies as reported by Harrison et al.; on the contrary, the OER appears to be slightly larger for these high energy neutrons than for the

Figure 1: Survival data for aerated and hypoxic Chinese hamster V79 cells exposed to graded doses of neutrons produced by bombarding a target of beryllium and tantalum with 80 MeV deuterons. The curves were drawn by eye.

Figure 2: Survival data for aerated and hypoxic Chinese hamster V79 cells exposed to graded doses of neutrons produced by bombarding a beryllium target with 101 MeV protons.

neutron beams currently in use in the clinic. On the grounds of the oxygen effect, therefore, there would appear to be no advantage to recommending higher energies for future clinical neutron facilities.

References

- Hall, E.J., Geard, C.R., Coffey, R.J., and Hall, B.E., 1977. Measurements of the oxygen enhancement ratio for high energy neutrons at the Fermilab. *International Journal of Radiat. Oncology, Biol., and Physics* 2:105-110.
- Hall, E.J., Geard, C.R., Povlas, S., and Astor, M., 1977. The oxygen enhancement ratio for high energy neutrons. *Brit. J. Radiol.* 50:679-680.
- Hall, E.J. and Kellerer, A.M., 1979. Review of RBE data for cells in culture. In *Proceedings of the Third Meeting on Fundamental and Practical Aspects of the Application of Neutrons and Other High LET Particles in Clinical Radiotherapy*. The Hague, The Netherlands, September 12-15, 1978. *Europ. J. Cancer*, In press.
- Hall, E.J., Lehnert, S., and Roizin-Towle, L., 1974. Split-dose experiments with hypoxic cells. *Radiology* 112:425-430.
- Hall, E.J., Roizin-Towle, L., Theus, R.B., and August, L.S., 1975. Radiobiological properties of high-energy cyclotron-produced neutrons used for radiotherapy. *Radiology* 117:173-178.
- Harrison, G.H., Kubiczek, E.B., Robinson, J.E., 1975. OER of neutrons from 80 MeV deuterons on beryllium. *Brit. J. of Radiol.* 48:409-410
- Harrison, G.H., Kubiczek, E.B., Robinson, J.E., 1976, OER reduction with high-energy neutrons. *Brit. J. Radiol.* 49:733.

Eric J. Hall, Myles Astor, Barbara Alonso and Steven Abrams

It has been demonstrated in a wide range of biological systems that hypoxic cells are relatively resistant to killing by x and γ -rays. The suggestion has been made frequently that the presence of viable hypoxic cells in human tumors may limit their curability by conventional radiotherapy, and as a consequence substantial effort has been devoted to developing methods to overcome this problem. The most recent method involves the use of hetero-cyclic nitro compounds that interact with radiation to increase specifically the sensitivity of hypoxic cells, while not affecting the response of normal aerated cells.

A number of compounds have been identified and studied in experimental systems and the most promising of them, misonidazole, has already been introduced into clinical trials. In addition to preferentially radiosensitizing hypoxic cells, misonidazole also exhibits a temperature-dependent cytotoxicity towards cells deficient in oxygen (1, 3, 6, 7, 8). The clinical use of misonidazole in radiotherapy patients is limited by the occurrence of peripheral neuropathy, and also symptoms arising from central nervous system damage, when large doses are given repeatedly. No firm correlation has been established between the neurological symptoms observed in patients and the cytotoxicity towards hypoxic cells shown with cells *in vitro* - but a connection is suspected.

Clinical trials of misonidazole are already well underway, but no definitive conclusions are likely for several years. Meanwhile, attention in the laboratory is directed towards identifying or synthesizing alternative compounds, which equal misonidazole in radiosensitizing efficiency, while at the same time exhibiting less troublesome side-effects. Two of the most promising contenders are desmethylmisonidazole and SR 2508.

Experiments with cells cultured *in vitro* were designed to compare the properties of misonidazole with these two potential replacements.

MATERIALS AND METHODS

Standard cell culture techniques were used to grow Chinese hamster V79 cells in Gibco F10 culture medium, supplemented with 10% fetal bovine serum, penicillin and streptomycin. For experiments to test the cytotoxicity and radiosensitizing properties of the various compounds, cells were treated in suspension in glass ampules. Hypoxia was induced by metabolic depletion as a result of crowding a large number of cells into a small volume of medium. The method has been widely used and described in detail elsewhere (2). The essential steps are as follows: cells from a number of actively growing, partially confluent stock flasks were harvested by trypsinization, washed to remove excess trypsin, and the concentration of cells determined by counting a sample in a Coulter electronic cell counter. This cell suspension was then divided into several parts and the cell concentration in each adjusted to 2×10^6 cells/ml, adding one or another drug at an appropriate concentration according to the plan of the particular experiment. From each of the cell suspensions containing various concentrations of one or another drug, or no drug at all, series of glass ampules were filled by pipetting 1 ml of the cell suspension into each. These ampules were then handled in the following way to render the cells hypoxic. Each ampule was flushed with a gas mixture consisting of 95% nitrogen plus 5% CO₂ in order to displace the air above the cell suspension, before being heat-sealed and transferred to a water-bath at 37.5°C where it was continuously shaken and tumbled end-to-end to keep the cells in suspension. This procedure was maintained for one hour to allow the cells to scavenge the oxygen dissolved in the medium by normal respiration and metabolism. A parallel series of ampules was filled with cells at a concentration of 10^4 /ml; these were gassed

with a mixture of air and 5% CO₂ before being heat-sealed. Because of the lower number of cells, these ampules remained aerated throughout. For the periods of time used in these experiments, the plating efficiency for cells, aerated and hypoxic, remained high (typically 70%) and unaffected by the experimental conditions used.

Following this procedure the cells were irradiated with γ -rays, or subjected to various periods in the water bath at 37.5°C to assess cytotoxicity. The source of γ -rays was a ⁶⁰Cobalt teletherapy unit, directed vertically upwards so that the cells were irradiated through the bottom of the glass ampules. At a treatment distance of 40 cm the dose rate was computed to be 1.95 Gy/min. Heat treatments were carried out by immersing the ampules in a water bath at 37.5°C controlled $\pm$ 0.1°C; the ampules were continuously tumbled and shaken during this heat treatment to prevent attachment of the cells to the glass surface.

At the conclusion of all treatments in a given experiment, each ampule was agitated on a vortex mixer to resuspend the cells, after which it was opened and various aliquots of the cell suspension plated into Falcon tissue-culture flasks containing fresh growth medium. After incubation for 8 days at 37.5°C, cells were fixed and stained, and the number of macroscopic colonies counted by a projection technique.

RESULTS

The sensitizing effectiveness of the compound SR 2508 is illustrated by the data in Figure RB-7-1. Cells were exposed to graded doses of ⁶⁰Cobalt gamma-rays under aerated and hypoxic conditions, in the presence or absence of SR 2508 at various concentrations. The drug is evidently an efficient radiosensitizer.

Figure RB-7-2 shows the results of a typical experiment designed to compare directly the radiosensitizing properties of misonidazole, desmethylmisonidazole

Figure 1: Survival data for Chinese hamster V79 cells exposed to graded doses of ^{60}Co gamma-rays under aerated and hypoxic conditions in the presence or absence of various concentrations of the compound SR 2508.

Figure 2: Survival data for Chinese hamster V-79 cells exposed to graded doses of ^{60}Co gamma-rays under aerated and hypoxic conditions in the presence or absence of a radiosensitizer SR 2508, Misonidazole or desmethylmisonidazole.

and SR 2508. The data were obtained in a large self-contained experiment in which all three drugs, at two different concentrations, were compared using cells from a common suspension. These, and other data not shown, indicate that the three drugs are virtually indistinguishable as far as radiosensitizing properties are concerned.

The cytotoxicity of misonidazole, desmethylmisonidazole and SR 2508 and were likewise compared in a series of large self-contained experiments. Cells made hypoxic by metabolic depletion were treated with a given concentration of the drug for various periods of time at 37.5°C and the survivors assayed by colony formation. The results of one such experiment is shown in Figure RB-7-3, in which the three drugs were compared at a concentration of 2 mM. It was found that misonidazole and desmethylmisonidazole were similar in their cytotoxicity, but that SR 2508 was substantially and significantly less cytotoxic in every experiment performed and at all concentrations from 1 to 5 mM. Also shown in in Figure RB-7-3 (open symbols) are survival data for cells derived from a single clone isolated from the survivors of a SR 2508 treatment, and subsequently retreated with the same drug one week later. It appears that pretreatment leads to an increased sensitivity to this drug.

DISCUSSION

A bewildering array of electron affinic compounds have been synthesized as alternatives to misonidazole. The choice of the next radiosensitizer to be tested in clinical trials is not obvious, but depends upon a number of factors. The compounds compared in the experiments described above are leading contenders.

Both desmethylmisonidazole and SR 2508 have shorter half-lives *in vivo* than misonidazole, so that reduced toxic side effects might be expected for a given degree of radiosensitization. This expectation is based on the premise that,

Figure 3: Survival data for Chinese hamster V79 cells exposed for various periods of time under hypoxic conditions to a 2 mM concentration of Misonidazole, desmethylmisonidazole or SR 2508. The open symbols refer to cells from a single clone isolated from the survivors of a previous SR 2508 treatment and subsequently retreated with the same drug.

while radiosensitization depends on peak serum concentration, toxic side effects are some function of the product of concentration and overall time. Both compounds proposed as alternatives to misonidazole are also more water-soluble and less lipid-soluble. Consequently, the concentration in the brain would be less for a given serum concentration and this might be expected to result in reduced neurological effects.

The experiments reported here involve comparisons between misonidazole, desmethylmisonidazole and SR 2508, using cells cultured *in vitro*. Factors such as the half-life *in vivo* and the partition coefficient play no part. It is evident from Figure RB-7-2 that the three compounds are virtually indistinguishable as radiosensitizers. On the other hand the cytotoxicity that they express towards hypoxic cells differs markedly. SR 2508 is appreciably less cytotoxic than the two other compounds, which result in similar levels of cell killing. There is some evidence from a limited number of experiments performed that cells pre-treated with SR 2508 are much more sensitive to subsequent treatment. The possibility of residual injury in surviving cells is a topic that clearly needs to be investigated further. Another parameter of importance is the carcinogenic potential of these heterocyclic nitro compounds. Miller and Hall (4) showed that misonidazole effectively produces oncogenic transformations *in vitro*. More recently it was found that, at a concentration of 1 mM, SR 2508 produced a transformation incidence about six times larger than either misonidazole or desmethylmisonidazole (5).

In conclusion, these *in vitro* studies show that the three 2-nitroimidazoles compared are virtually indistinguishable as radiosensitizers. At the same time, SR 2508 is substantially less cytotoxic towards hypoxic cells than misonidazole or desmethylmisonidazole, but is probably a more potent carcinogen. These

relative properties, as well as the half-life *in vivo* and the partition coefficient must be taken into account in choosing the replacement for misonidazole that will enter the costly and time consuming phase of clinical trials.

References

- 1) Hall, E.J., Astor, M., Geard, C. and Biaglow, J., 1977. Cytotoxicity of Ro-07-0582; enhancement by hyperthermia and protection by cysteamine. Br. J. Cancer 35:809-815.
- 2) Hall, E.J., Lehnert, S., and Roizin-Towle, L., 1974. Split-dose experiments with hypoxic cells. Radiol. 112:425-430.
- 3) Hall, E.J. and Roizin-Towle, L., 1975. Hypoxic sensitizers: radiobiological studies at the cellular level. Radiol. 117:453-457.
- 4) Miller, R.C. and Hall, E.J., 1978. Oncogenic transformation *in vitro* by the hypoxic cell sensitizer misonidazole. Br. J. Cancer 38:411-417.
- 5) Miller, R.C. and Hall, E.J., 1980, Oncogenic transformations *in vitro* produced by misonidazole. Cancer Clinical Trials 3:85-90.
- 6) Mohnidra, J.E. and Rauth, A.M., 1976. Increased cell killing by metronidazole and nitrofurazone of hypoxic compared to aerobic mammalian cells. Cancer Res. 36:930-936.
- 7) Stratford, I.J. and Adams, G.E., 1977. Effect of hyperthermia on differential cytotoxicity of a hypoxic cell radiosensitizer Ro-07-0582 on mammalian cells *in vitro*. Br. J. Cancer 36:807-813.
- 8) Sutherland, R.M., 1974. Selective chemotherapy of noncycling cells in an *in vitro* tumour model. Cancer Res. 34:3501-3503.

RB-8 In Vitro studies with cis-platinum diamide dihydrochloride and exogenous polyamines.

L. Roizin-Towle and Louis Capuano

INTRODUCTION

The role played by polyamines in cell growth and metabolism is an intensive area of current research. Their existence has been known for several years, but it is their function as growth promoters that has most recently raised interest as to their possible association with neoplastic processes (Diane Russell, 1973). Elevated levels of polyamines are associated with cellular growth, i.e., embryogenesis, hepatic regeneration and lactation. They are also present in elevated concentrations in the urine and sera of patients bearing malignant tumors (Harik and Sutton, 1979; Marton et al., 1973). The finding by Russell and subsequently by others that polyamine levels increase during the malignant state, has singled them out as potential biological markers for cancer detection. Whether increased polyamine activity is directly related to tumor growth or a consequence of it remains uncertain. Their presence, however, and that of their regulating enzymes during the cancer process appear to be intimately related. This is further supported by evidence showing increased levels of ornithine decarboxylase (the regulating enzyme for putrescine production) found with oncogenic transformation *in vitro*.

Among the naturally occurring polyamines putrescine, spermine and spermidine are the best known. Their levels increase during growth and differentiation and these increases usually precedes increases in RNA, DNA, and protein synthesis. It is believed that they stabilize secondary structures of DNA because they are able to bind tightly to nucleic acids. Their levels naturally decline when cell

division or differentiation is completed.

Since many human cancer patients receive chemotherapy agents at some time during the management of their disease, it seemed pertinent and relevant to ask whether or not the action of these agents was affected by the presence of various levels of polyamines. Cis diamminedichloro-platinum, (Cis-DDP) an antineoplastic agent used primarily in the treatment of testicular, ovarian and bladder carcinomas, was used for the studies reported here.

Materials and Methods

Chinese hamster V-79 cells were used for these experiments. Standard culture techniques were used, with the cells grown in GIBCO F10 culture medium supplemented with 10% fetal calf serum and antibiotics. Under these conditions routine growth curves indicated a population doubling time of approximately 10 hours. For all experiments cells were harvested from stock cultures by trypsinization and counted with an electronic particle counter (Coulter Electronics, Hialeah, Florida).

After appropriate dilutions, sufficient cells were inoculated with 25 cm^2 Falcon plastic tissue culture flasks so that an estimated number of about 100 viable cells per flask would survive the planned drug treatment. The number of cells per flask was limited to the range where surviving fraction is not influenced by the size of the inoculum. Cells plated in the late afternoon were treated with drug combinations the following morning, the time interval allowing cells to attach and enter an experimental phase of growth. Replicate flasks were exposed either to Cis-DDP, polyamines or a combination of both.

Subsequent to drug(s) exposure, flasks were rinsed with Puck's saline, replenished with fresh media, and kept for an 8 day period in a 37.5°C incubator to allow for colony formation.

Drug

Cis-DDP was obtained from the Drug Synthesis and Chemistry Branch, Division of

Cancer Treatment, National Cancer Institute. Polyamines were purchased from the Sigma Chemical Company, St. Louis, Missouri. For all experiments polyamines and Cis-DDP were initially dissolved in Hanks Balanced Salt Solution and diluted with complete media previous to cell exposure. Drug suspensions were made up fresh the same day of the experiment.

Results

Survival data for V-79 cells exposed at 37.5°C to various concentrations of Cis-DDP for 1 to 6 hours are shown in Figure 1. An exposure to 100 uM of Cis-DDP for one hour reduces cell survival by a factor of 10^3 , whereas by 6 hrs a 20 uM exposure to drug results in the same amount of cell killing. Figure 2 shows the effect of a two hour simultaneous treatment with Cis-DDP and putrescine. The presence of the polyamine considerably reduces the cytotoxicity of the Cis-DDP, even at a concentration of putrescine that was non-toxic to the cells. Figure 3 shows the data for cells treated for a range of exposure times with a constant dose of Cis-DDP (20 uM). In this case treatment with Cis-DDP alone was compared to treatments with Cis-DDP and two concentrations of putrescine. Both concentrations of polyamine (10 and 30 mM) protect against platinum toxicity for all time periods tested with the higher concentration of putrescine being the more effective. The data in Figure 4 show how the temporal sequence of putrescine application effects subsequent platinum toxicity. As in Figure 2, cells were treated for 2 hours with various concentrations of Cis-DDP. To assess the optimum effectiveness of putrescine, cells were treated for 2 hours with 30 mM putrescine before, during or after exposure to platinum. Clearly, putrescine is most effective when present at the same time as platinum treatment.

The next two figures, Figs. 5 and 6, show the result of experiments with another of the polyamines, spermine. Spermine exhibits considerable toxicity *in vitro* if

Figure 1. Survival data for V-79 hamster cells at 37.5°C for various time periods with Cis-DDP.

Figure 2. Survival data for V-79 hamster cells treated at 37.5°C with increasing doses of Cis-DDP in the presence or absence of putrescine.

Figure 3. Survival data for V-79 hamster cells treated at 37.5°C for various time periods with a constant dose of Cis-DDP, with or without putrescine.

Figure 4. Illustrating the effect of the sequence of treatments with putrescine and Cis-DDP.

Figure 5. Survival data for V-79 hamster cells showing the toxic effects of spermine on cells in medium containing fetal calf serum. The toxicity is reduced in the absence of serum.

Figure 6. Survival data for V-79 cells treated for 2 hours at 37.5°C with Cis-DDP alone, or in combination with spermine. Serum-free medium was used to avoid the problem of toxic oxidation products formed by the interaction of spermine with fetal calf serum.

fetal calf serum is present in the culture media. Amine oxidase, present in calf serum, oxidizes spermine to a toxic product, aminoaldehyde. This is illustrated in Fig. 5. With cells in media including 10% fetal calf serum there is considerable toxicity with increasing polyamine concentrations. This toxicity is greatly reduced if the treatments are carried out in serum-free growth medium. Experiments treating V-79 cells with Cis-DDP in complete and incomplete media (no serum) showed that the toxicity to platinum was virtually the same with or without serum present (not shown). In order to study the effects of spermine *in vitro* and avoid toxicity due to the serum oxidation products, experiments have to be done in medium without serum or media pre-treated with aminoguanidine, an analogue of spermine which inactivates amine oxidase. In these experiments, spermine treatments were performed in incomplete media to avoid this problem.

Figure 6 shows the result of a 2 hour exposure of cells to increasing platinum concentrations with and without the presence of spermine in incomplete media. Like putrescine, spermine also protects against Cis-DDP toxicity.

Discussion

Cis-Diamminedichloroplatinum is an established chemotherapy agent (W.E. Heydorn, 1979). It is used predominantly in the treatment of ovarian, testicular and bladder carcinomas, as well as tumors of the head and neck. The cytotoxic effect of Cis-DDP results from interaction with DNA or its constituents. This interaction with DNA occurs by monofunctional binding to bases in the helix, either on the same strand or opposite strand (Douple, 1979). The cytotoxicity of this drug falls into four categories: renal, auditory, gastrointestinal and miscellaneous. Since cytotoxicity is ultimately the first and last concern in any drug therapy program, this was the focal point of this study. Traditionally, *in vitro* studies of drug toxicities are studied by exposing cells to various concentrations of drug for various periods of time and scoring cell survival. This is the counterpart to *in vivo* studies where

toxicity is measured by tumor regrowth delay. In either case, there are other factors which may alter the final outcome of toxicity, but these are limited by the system being used.

The rationale for studying the combined effects of Cis-DDP and polyamines is based on an increasing body of evidence suggesting elevated levels in the plasma of cancer patients (Russel et al., 1975). They are endogeneous amino-acid intermediates intimately involved with the growth process whether it be normal or neoplastic (Ham, 1964; Duffy et al., 1971). Their role in cell growth, metabolism and protein synthesis has been studied for years. Since their levels may be significantly elevated during the course of tumor growth, their presence may influence the cytotoxic effects of chemotherapy drugs used in cancer treatment. The basis of action for most chemotherapeutic agents is primarily their interference with DNA synthesis. Polyamines may alter this effect as they are thought to stabilize secondary structures of nucleic acids. Figures 2, 3, and 6 demonstrate the extent to which putrescine and spermine inhibit the cytotoxicity of the chemotherapy drug Cis-DDP. The data demonstrate convincingly that for this particular antineoplastic agent, cytotoxicity is reduced by elevated levels of polyamine. This protection occurs at a level of Cis-DDP that is within the clinical dosage (Drewinko et al., 1973). It was shown further that the interaction between putrescine and Cis-DDP varies only when the two compounds are present simultaneously. The mechanism of this interaction is unknown.

The toxic effects of fetal bovine serum and spermine was demonstrated in Fig. 5. This has been shown by others (Webber and Chaproniere-Rickenberg, 1980; Tabor et al., 1964) and is unique to ruminant serum. Nevertheless, spermine was shown, likewise to protect against Cis-DDP toxicity (Fig 6).

The results presented here raise questions as to the effect(s) polyamines exert in combination with chemotherapy agents. Since they are naturally occurring

substances in mammalian cells which play a definite role in growth promotion and regulation, their interaction with chemotherapy agents warrants further investigation.

References

1. Duffy, P.E., Defendini, R., and Kremzner, L.T. Regulation of Meningioma Cell Growth *In Vitro* by Polyamines, *J. Neuropath Exp Neurol* 30: 698-713, 1971.
2. Drewinko, B., Brown, W., and Gottlieb, J.A. The Effect of Cis-Diamminedichloroplatinum (II) on Cultured Human Lymphoma Cells and Its Therapeutic Implications, *Cancer Res* 33: 3091-3095, 1973.
3. Douple, E.B. and Richmond, R.C. A review of platinum biochemistry suggests a rationale for combined platinum-radiotherapy, *Int'l J. Radiat. Oncol Biol Physics* 5: 1335-1340, 1979.
4. Ham, R.G. Putrescine and related amines as growth factors for a mammalian cell line, *Biochem Biophys Res Commun* 14: 34-38, 1964.
5. Harik, S.I. and Sutton, C.H. Putrescine as a biochemical marker of malignant brain tumors, *Cancer Res* 39: 5010-5015, 1979.
6. Heydron, W.E. A clinical review of cisplatin, *Hosp Phar* 14: 316-329, 1979.
7. Marton, L.J., Vaughn, J.G., Hawk, I.A., Levy, C.C., and Russell, D.H. Elevated polyamine levels in serum and urine of cancer patients. In: *Polyamines in Normal and Neoplastic Growth*, Raven Press, Publishers; New York, 1973.
8. Russell, D.H. (Ed) *Polyamines in Normal and Neoplastic Growth*. Raven Publishers New York, 1973.
9. Russell, D.H., Durie, B.G.H., and Salmon, S.E. Polyamines as predictors of success and failure in cancer chemotherapy, *Lancet* 2: 797-799, 1975.
10. Tabor, C.W., Tabor, H., and Backrack, U. Identification of the aminoaldehydes produced by the oxidation of spermine and spermidine in purified plasma amine oxidase, *J Biol Chem* 239: 2194-2203, 1964.
11. Webber, M.M. and Chaproniere-Rickenberg. Spermine oxidation products are selectively toxic to fibroblasts in culture of normal human prostatic epithelium, *Cell Biol Intern Rep* 4: 185-193, 1980.

RB-9 The development and decay of thermotolerance.

M.L. Freeman

The relationship between cell inactivation and the duration of a heat treatment is sigmoidal in shape in most cases (1). For many cell lines, however, when heating is continued beyond 200 to 300 minutes, cells become refractory to killing by heat, the survival curve becomes shallower and loses its sigmoidal shape (2,3). The phenomenon has been described as "thermotolerance", and is only observed at temperatures below 43°C (4).

Little is known about the mechanisms which cause mammalian cells to become thermotolerant. This phenomenon is not seen in all cell lines. For example, Raaphorst et al. (5) heated 7 cell lines *in vitro* at 42.5°C and found that tolerance was transitory in rat kangaroo cells, but failed to develop at all in mouse LP 59 cells. On the other hand, Chinese hamster ovary, V-79, HeLa S3, pig kidney, and muntjac cells all exhibited thermotolerance. Various environmental parameters can affect tolerance. For example, heating CHO cells in hypotonic medium (0.1 M or 0.075 M) caused thermotolerance to decay after it developed (6). Heating cells in an acidic environment did not prevent the development of tolerance but reduced the proportion of cells which exhibit it compared to cells heated in an alkaline environment (7).

The present study was initiated to investigate the development and decay of thermotolerance, in the hope that a better understanding of the mechanism of hyperthermic induced cell killing may be achieved.

METHODS AND MATERIALS

Chinese hamster ovary (CHO) cells growing exponentially in monolayer cultures were maintained at 37°C at a pH of about 7.4 in McCoy's 5a medium containing either 10% fetal calf serum (Figs. RB-9-1 & RB-9-3) or 20% heat in-

Figure 1. The development and decay of thermotolerance in CHO cells. ▲ less than 1500 cells inoculated into T-25 flasks; Δ - 1500 cells inoculates; □ - 1×10^4 cells inoculated; ◇ - 1×10^5 cells inoculated.

Figure 2. The development and decay of thermotolerance in V-79 cells. $\blacktriangle$ - less than 1500 cells inoculated into T-25 flasks; $\triangle$ - 1500 cells inoculated; $\square$ - 1×10^4 cells inoculated; $\diamond$ - 5×10^4 cells inoculated.

Figure 3. The effect of temperature on the decay of thermo-tolerance in CHO cells. CHO cells were heated at 41.9°C or 41.4°C .

activated calf serum (Figs. RB-9-4 & RB-9-5), plus 100 units/ml of penicillin G sodium and 100 mcg/ml of streptomycin sulfate. Chinese hamster V-79 cells were maintained similarly in F-10 (Ham) culture medium containing 10% fetal calf serum and supplemented with 426 units/ml of penicillin G sodium, 426 mcg/ml streptomycin, and 0.3 mg/ml L glutamine.

Hyperthermic treatment commenced 15-24 hours after cells had been inoculated into Falcon T-25 flasks. This procedure allowed the cells to attach and enter an exponential phase of growth prior to treatment. Heat treatments were conducted in GCA Precision Scientific water baths. The temperature was monitored using an NBS calibrated thermometer, so that all stated temperatures were within $\pm 0.05^{\circ}\text{C}$.

Survival determinations were corrected for both plating efficiency and multiplicity ($\bar{N}$) (8).

Preliminary experiments were carried out to check that extended heating did not denature the medium, and it was found that heating McCoy's 5A or F-10 medium for up to 72 hours at 42.0°C did not affect the plating efficiency. Incubating V-79 or CHO cells for up to 72 hrs in a 37°C water bath also did not affect the plating efficiency (results not shown).

RESULTS

Figure RB-9-1 illustrates that for CHO cells thermotolerance was transient in nature. After 4 hours of heating at 41.9°C cells became thermotolerant, i.e., the D_0 was increased from 2 to 30 hours. The "thermotolerant state" lasted for 32 hours and then decayed in the final 36 hours of heating ($D_0 = 9$ hrs). These results were independent of the number of viable CHO cells treated. In contrast, the survival of V-79 cells was dependent upon cell number, Figure RB-9-2. Similar to CHO cells the survival curve had three components but the final slope decreased as the number of viable cells treated was increased.

Figure 4. The effect of temperature on the decay of thermo-tolerance in CHO cells heated at 41.8°C or 41.5°C.

Figure 5. The effect of temperature on the percent of cells in mitosis. A minimum of 1000 cells were counted per point. 0, ● - exp 45; ▽ △ ▲ - exp 46; ▽ 42.0°C; 0, △ - 41.4°C, ●, ▲ - 37°C.

To test whether the decay of thermotolerance was associated with cell progression (i.e., cells moving from a resistant phase of the cell cycle to a sensitive one) CHO cells were heated at two different temperatures. Cells would be expected to progress more rapidly at the lower temperature thus moving into the sensitive phase faster than cells heated at the higher temperature. This would result in a greater cytotoxicity for the lower than for the higher temperature after a critical heating period had elapsed sufficient for reassortment of cells within the cycle. This is consistent with data shown in figures RB-9-3 and RB-9-4, where following a period of thermotolerance, a lower temperature kills more cells than a higher temperature. In all cases in Figures RB-9-3 and RB-9-4, the dose response relationships have three components, with the duration of the thermotolerant state shortening with decreased temperature.

Figure RB-9-5 shows data from two experiments in which mitotic index was monitored as a function of heating time in parallel with determinations of cell survival. Cell survival is not shown but was qualitatively similar to that seen in Figure RB-9-3. At 41.4°C the M.I. curve could be characterized by 3 components: an initial drop, an accumulation of cells in mitosis, and finally a steady progression of cells through mitosis. At 42.0°C the shape of the M.I. curve was qualitatively similar to that seen at 41.4°C, but the percent of cells in mitosis was much lower, e.g., after 8 hours of heating the M.I. was 0.65%. These studies of the mitotic index suggest that cells progressed through mitosis at both temperatures but the rate was slower at 42.0°C.

DISCUSSION

The data reported here, as well as similar findings of Read, Fox, and Bedford (9) illustrate that thermotolerance in V-79 and CHO cells is transient in nature. The experiments illustrated in Figures RB-9-1 to RB-9-4 are also con-

sistent with the work of Sapareto et al. (3) and Palzer and Heidelberger (2). These investigators showed that if thermotolerance developed it occurred after 200-300 minutes of heating at or below 42.5°C. Furthermore, the work of Highfield et al. (10) has been extended by this study to show that the rate of decay of thermotolerance in V-79 cells was dependent upon cell number.

As suggested above, the decay of thermotolerance may be associated with cell progression through the cycle. This is an attractive hypothesis and is consistent with the experimental data. An alternative explanation may be that the degree of thermotolerance increases as the level of survival is decreased. This was pointed out by Leith et al. (11) for thermotolerance developing during fractionated hyperthermia, and it is possible that the duration of the thermotolerant state seen in Figure RB-9-1 to RB-9-4 may be due to the same course. Above 41.6°C the final slope of the survival curve was less than the initial slope indicating that the decay of thermotolerance was not complete. At 41.4°C the initial and final slopes were very similar although the assessment of the initial slope may be complicated by the development of thermotolerance. If the decay of thermotolerance is associated with cell progression, then hyperthermic induced cellular inactivation would be cell cycle specific even when the cells are in a thermotolerance state. On the other hand if the degree of thermotolerance is a function of survival level then the thermotolerant inactivation rate would be independent of cell cycle parameters. Further work is needed before this problem can be resolved.

References

- 1) Harris, M., 1967. Temperature-resistant variants in clonal population of pig kidney cells. *Exp. Cell Res.* 46:301-314.
- 2) Palzer, R.J., Heidelberger, C., 1973. Studies on the quantitative biology of hyperthermic killing of HeLa cells. *Cancer Res.* 33:415-421.
- 3) Sapareto, S.A., Hopwood, L.E., Dewey, W.C., Raju, M.R., Gray, J.W., 1978. Effects of hyperthermia on survival and progression of Chinese hamster

ovary cells. Cancer Res. 38:393-400.

- 4) Dewey, W.C., Hopwood, L.E., Sapareto, S.A., Gerweck, L.E., 1977. Cellular responses to combinations of hyperthermia and radiation. Radiology 123:463-474.
- 5) Raaphorst, G.P., Romano, S.L., Mitchell, J.B., Bedford, J.S., Dewey, W.C., 1979. Intrinsic differences in heat and/or x-ray sensitivity of seven mammalian cell lines cultured and treated under identical conditions. Cancer Res. 39:396-401.
- 6) Raaphorst, G.P., Dewey, W.C., 1978. Enhancement of hyperthermic killing of cultured mammalian cells by treatment with anisotonic NaCl or medium solutions. J. Thermal Biol. 3:177-182.
- 7) Freeman, M.L., Raaphorst, G.P., Hopwood, L.F., Dewey, W.C., The effect of pH on cell lethality induced by hyperthermic treatment. Cancer 45: 2291-2300 (1980).
- 8) Elkind, M.M., Whitmore, G.F., 1967. The radiobiology of cultured mammalian cells. Gordon & Breach, New York, N.Y., p. 41.
- 9) Read, R.A., Fox, M.H., Bedford, J.S., Some effects of chronic hyperthermia on three different cell lines. Abstracts of the papers for the 28th annual meeting of the Radiation Research Society. New Orleans, Louisiana, 1-5 June 1980.
- 10) Highfield, D.P., Holahan, E.V., Dewey, W.C., Effects of cell density on the survival response of CHO cells treated by hyperthermia. Abstracts of papers for the 28th annual meeting of the Radiation Research Society. New Orleans, Louisiana, 1-5 June 1980.
- 11) Leith, J.T., Miller, R.C., Geiner, E.W., Boone, M.L.M., 1977. Hyperthermic potentiation. Biological aspects and applications to radiation therapy. Cancer 39:766-779.

RB-10 Sensitivity to x-rays and UV of cis platinum (II) sensitive
V-79 cells.

M. L. Freeman

INTRODUCTION

A striking aspect of the chemotherapeutic drug cis platinum (II) diammine-dichloride (cis Pt II) is that its cytotoxicity varies considerably among different mammalian cell lines. Differences in both the accumulation of damage during an exposure (i.e., the amount of platinum which binds to chromatin, for review see 1 and 2) and the efficiency of repair systems can explain varying sensitivities as well as complicate a comparison of platinum induced cytotoxicity in different cell lines. For example, HeLa cells were found to be 3.3 times as sensitive to treatment with cis Pt (II) as V-79 cells when survival was compared on an exposure basis (3). However, twice as much platinum was bound to the HeLa cells' DNA for a given dose of the drug. When the increased platinum binding was corrected for the HeLa cells were only 1.55 times as sensitive. Analogous results were found when normal FL/A human fetal lung cells and UV repair deficient Xeroderma Pigmentosum XP-12-BE cells (complimentation group A) were exposed to cis Pt (II) (4). On an exposure basis the XP cells were 1.5 times as sensitive as FL/A cells. After correcting for the fact that 3 times more platinum bound to FL/A cells, the XP cell line was found to be 4 times as sensitive as the FL/A cell line.

In order to gain a better understanding of the parameters which influence comparisons of cis platinum induced cytotoxicity, an attempt was made to isolate a cell strain resistant to cis Pt (II) using a technique similar to the one Levis used to isolate a cell strain resistant to nitrogen mustard (5). In that study

RCP guinea pig cells were treated with a single dose of 20 $\mu\text{g/ml}$ of HN_2 , which reduced survival to 2×10^{-5} . A resistant subline was isolated from the survivors of that treatment. In the work reported here a sensitive rather than a resistant cell line was isolated.

MATERIALS AND METHODS

Methods of cell culture

Chinese hamster V-79-1 and V-79-2 cells were maintained as an exponentially growing monolayer in a humidified 37°C incubator with a controlled atmosphere of 95% air and 5% CO_2 , using F-10 (Ham) culture medium supplemented with 426 units/ml of penicillin G sodium, 426 mcg/ml streptomycin sulfate, 0.3 mg/ml L glutamine, and 10% serum. In the experiments illustrated in Figures RB-10-1, RB-10-2, and RB-10-5, and Table RB-10-1, 10% fetal bovine serum was used, while in Figs. RB-10-3 and RB-10-4, 7% calf and 3% newborn calf sera were used (due to the unavailability of fetal bovine serum). In 10% fetal calf serum the population doubling time was 9-12 hours and in 3% newborn -7% calf sera it was 14-16 hours. Both V-79-1 and V-79-2 cells are aneuploid and have a chromosome complement that ranges between 23-25 chromosomes. The lines were passaged three times a week.

Drug treatment

Experiments were initiated by inoculating Falcon T-25 flasks with the appropriate number of cells to yield approximately 100 surviving colonies. After overnight incubation (15 -24 hours) at 37°C to allow cells to attach and enter an exponential phase of growth, the growth medium was removed by aspiration and replaced with full medium containing freshly dissolved cis PT (II) (NSC 119875). The controls were handled similarly except that no drug was used. All dishes were then incubated at 37°C for 2 hours, after which the medium was removed, the dishes were washed twice with Pucks Saline F, and fresh growth medium was added with or

TABLE RB-10-1

Cell	Dose (Gy)	Survival ($\pm$ SEM)
V-79-1	0	0.83
	3	0.22 ($\pm$ 0.01)
	5	0.088($\pm$ 0.004)
	12	0.0068 ($\pm$ 0.0004)
V-79-2	0	0.78
	3	0.24 ($\pm$ 0.02)
	5	0.096 ($\pm$ 0.01)
	12	0.0046 ($\pm$ 0.0003)

The survival of V-79-1 and V-79-2 cells (passage 34) X-irradiated at room temperature. $\bar{N}$ was 4.59 and 3.96 for V-79-1 and V-79-2 cells respectively.

Figure 1. The cytotoxic action of cis Pt(II) on V-79-1 cells (Δ) and V-79-2 cells (O, Passage 6). The plating efficiency was 1.3 or 0.60 and $\bar{N}$ was 3.2 or 2.5 for V-79-1 and V-79-2 cells respectively. Standard error of the mean is indicated only when larger than the symbol in this and all other figures. Δ - $D_0 = 6.5 \mu\text{M}$ and $D_q = 7.5 \mu\text{M}$; O - $D_0 = 5.5 \mu\text{M}$ and $D_q = 1 \mu\text{M}$. D_q is the "quasithreshold dose."

Figure 2: The enhancement of platinum damage by 0.75 mM caffeine in V-79-1 cells (Δ , Δ) and V-79-2 cells ($\circ$, $\circ$; 3rd passage after thawing). Open symbols are the same as in Fig. 1. Closed symbols represent cells treated with platinum and then placed in full medium containing 0.75 mM caffeine for colony formation. The plating efficiency of V-79-1 cells was 0.57 in the absence of caffeine and 0.59 in the presence of caffeine; $\bar{N} = 3.4$. The plating efficiency of V-79-2 cells was 0.68 in the absence and 0.65 in the presence of caffeine; $\bar{N} = 3.3$. Δ - $D_0 = 8.5 \mu\text{M}$ and $D_q = 3.5 \mu\text{M}$; $\blacktriangle$ - $D_0 = 3.5 \mu\text{M}$ $D_q = 6.5 \mu\text{M}$ and $\circ$ - $D_0 = 5.8 \mu\text{M}$ and $D_q = 6 \mu\text{M}$; $\bullet$ - $D_0 = 2.25 \mu\text{M}$ and $D_q = 3.5 \mu\text{M}$.

Figure 3 The survival of V-79-1 (Δ , $\blacktriangle$) and V-79-2 cells ($\circ$, $\bullet$; 26th passage after thawing) UV irradiated and then grown in the absence (open symbols) or presence (closed symbols) of 0.75 mM caffeine. The plating efficiency of V-79-1 cells was 0.66 and 0.67 in the absence and presence of caffeine; $\bar{N} = 2.5$. The plating efficiency of V-79-2 cells was 1.01 and 0.85 in the absence and presence of caffeine; $\bar{N} = 2.3$.

Figure 4 The enhancement of platinum damage by 0.75 mM caffeine in V-79-1 and V-79-2 (31st passage after thawing) cells. The symbols are the same as in Fig. 2. The plating efficiency of V-79-1 cells was 0.55 and 0.44 in the absence and presence of caffeine; $\bar{N} = 1.3$. The plating efficiency of V-79-2 cells was 0.51 and 0.28 in the absence and presence of caffeine; $\bar{N} = 1.4$. Δ - $D_0 = 8 \mu\text{M}$ and $D_q = 1.5 \mu\text{M}$; $\blacktriangle$ - $D_0 = 3 \mu\text{M}$ and $D_q = 3 \mu\text{M}$; $\circ$ - $D_0 = 5 \mu\text{M}$ and $D_q = 4 \mu\text{M}$; $\bullet$ - $D_0 = 2 \mu\text{M}$ and $D_q = 1.5 \mu\text{M}$.

Figure 5 The cytotoxicity of cis Pt(II). Closed symbols represent the cell survival of V-79-1 cells exposed to a dose of 65 μ M cis Pt and then reexposed to the indicated doses 11 days after the first treatment. Open symbols represent the survival of V-79-1 cells exposed only to the indicated doses of cis Pt (II) (i.e., these cells were not pretreated). $\diamond$ - PE = 0.65, $\bar{N}$ = 2.97; $\blacklozenge$ - PE = 0.62, $\bar{N}$ = 3.0; $\square$ - PE = 0.68, $\bar{N}$ = 4.33; $\blacksquare$ - PE = 0.77, $\bar{N}$ = 3.98

without 0.75 mM caffeine. The dishes were then placed in a humidified 37°C incubator for colony formation. Seven to fifteen days later the colonies were stained with Giemsa and counted. Survival was corrected for both plating efficiency and multiplicity ($\bar{N}$). (6)

Preliminary experiments were carried out to see if the rinsing procedure or the number of cells inoculated into a flask affected survival. The rinsing procedure did not affect the number of surviving colonies nor was survival affected by cell number provided the cell inoculum was kept below 1×10^5 cells per flask (data not shown).

Methods of irradiation

UV light

Cells were inoculated into Falcon 60 mm petri dishes and allowed to attach overnight. Just prior to irradiation the growth medium was removed, the cells washed in Hanks Balanced Salt Solution (HBSS), and then irradiated in 1 ml of HBSS using a General Electric Germicidal lamp G-15-8 (principal wave length 254 nm) at a dose rate of 1 erg/mm^2 . Following irradiation, the HBSS was removed and full growth medium, with or without caffeine, was added to the cells to allow colony formation.

X-ray

Cells were allowed to attach overnight and x-irradiated in full growth medium using a Siemens Stabilipan x-ray machine operated at 300 kVp, 12 mA, with 0.5 mm Cu added filtration. The dose rate was computed to be 1.98 Gy/min.

RESULTS

The isolation of a cell line with increased sensitivity to cis platinum occurred only once in this series of experiments. These cells, designated V-79-2, are the progeny of V-79 cells which survived a dose of 65 μM cis PT (II)

administered for 2 hrs at 37°C. This protocol reduced survival to 1×10^{-5} . Twelve days after treatment all the viable cells (1-2 colonies) from a single T-25 flask were isolated, subcultured over an additional 9 days, and then replated as described in the Materials and Methods for retreatment with cis Pt (II) (Fig. RB-10-1). As seen in this figure, those cells pretreated with the drug (V-79-2) were more sensitive than cells which were not pretreated (V-79-1).

The sensitivity of the subline (V-79-2) to platinum induced damage was unaffected by changes in the population doubling time. Six passages after isolation, V-79-2 cells had a doubling time of 15 hours but after passage 8 the doubling time was identical with that of the parental line. The sensitivity of the subline was rechecked at the 22nd passage (data not shown) and the results were similar to those shown in Figure RB-10-1. The V-79-2 cells were frozen in liquid nitrogen after the 27th passage. The experiments represented in Figs. RB-10-2 through RB-10-4 were performed using cells thawed from the liquid nitrogen pool. The experiments in Fig. RB-10-1 and Table RB-10-1 were performed using cells that were never frozen.

A nontoxic concentration of caffeine, added after platinum treatment, potentiated cis Pt (II) cytotoxicity in both the parental and drug-sensitive cell lines (Fig. RB-10-2). Caffeine reduced the D_0 (the dose required to reduce survival by 1/e) by a factor of 2.4 in the parental line and 2.6 in the subline, indicating that the degree of synergism was the same for both lines at equal levels of cell killing.

The response of the two cell lines to UV irradiation was identical as shown in Fig. RB-10-3. Caffeine reduced the D_0 of the UV survival curve by a factor of 3 for both lines. It is interesting to note that even in the absence of caffeine the UV dose response curve lacked a shoulder.

The experiment shown in Fig. RB-10-4 was carried out to recheck the subline's sensitivity to platinum cytotoxicity 31 passages after thawing (five passages after the experiment depicted in Fig. RB-10-3) and to determine the dose response curves of the two lines when grown in calf sera and treated with platinum (see Materials and Methods). The results are similar to those shown in Fig. RB-10-2. Coincidental with the change to calf sera was the fact that 0.75 mM caffeine became toxic to V-79-2 cells. Caffeine reduced the plating efficiency of the subline by 16% in Fig. RB-10-3 and 45% in Fig. RB-10-4. Note that the dose response curves were corrected for caffeine toxicity.

The two cell lines are indistinguishable in their response to x-rays as shown by the data summarized in Table RB-10-1.

In general, however, the rate of cis Pt (II) induced cellular inactivation did not change after multiple treatments with the drug. Two experiments which illustrate this point are shown in Fig. RB-10-5. In these experiments V-79 cells were exposed to a dose of 65 μ M cis Pt (II) and all the surviving cells in 4 or 10 flasks (8 or 16 colonies per flask) were reexposed to the drug 11 days (12 doublings) after the first treatment. It is evident that no significant change in sensitivity occurred in a majority of the survivors over the period tested.

DISCUSSION

The experiments described here demonstrate that a stable cell line, V-79-2, isolated from V-79 cells which had survived a pretreatment with cis Pt (II) exhibited a greater susceptibility to cis Pt (II) induced damage when compared to the parental line. Yet both lines had the same population doubling time, chromosome complement, and the same sensitivity to X or UV irradiation.

Two reports (3,7) have indicated that in HeLa, V-79, and human lymphoma cells cis Pt (II) cytotoxicity varied with cell cycle phase. But it is unlikely that

the results seen in Figs. RB-11-1, RB-10-2, and RB-10-4 are due to differences in cell cycle parameters since the population doubling times of the two lines were the same.

Both cell lines appear to repair platinum induced damage to the same degree. Van den Berg and Roberts (8,9) have shown in V-79 cells that caffeine increased platinum cytotoxicity by inhibiting a post replication repair system. If V-79-2 cells were post replication repair defective then the degree of synergism seen when caffeine was added after platinum treatment would not have been equal to that seen in V-79-1 cells. Even if the subline was defective in a repair mechanism which was not caffeine sensitive, the degree of synergism should not be the same in the two lines. For this argument to be valid, caffeine must inhibit post replication repair of platinum damage to the same degree in both lines. Fig. RB-10-4 illustrates that caffeine potentiation of UV damage (10) was the same in both cell lines indicating that post replication repair of this damage (11) was equally inhibited. This suggests that caffeine does not act in a differential manner in the two cell lines.

In line with this argument Szumiel (12,13) has shown that in two strains of murine lymphoma L5178Y cells UV light and cis-dichlorobis (cyclopentylamine) platinum (II) (cis PAD) sensitivity were related. Interestingly, the strain which exhibited a greater resistance to UV irradiation also exhibited greater sensitivity to x-irradiation. The L5178YS cell line was comparatively more resistance to UV light or cis PAD cytotoxicity than the L5178YR line. Caffeine potentiation of cis PAD damage occurred only in the L5178YS line. The increased sensitivity of the L5178YR line was thought to be due partly to a diminished capacity for caffeine sensitive post replication repair.

This work illustrates that parameters other than repair of cis platinum

damage appear to account for the differential platinum sensitivity exhibited by the V-79-1 and V-79-2 cell lines. Furthermore, equal degrees of caffeine potentiation of platinum damage (Figs. RB-10-2 and RB-10-4) suggest that more platinum was present in the subline compared to the parental line for a given drug exposure. In these cells viability after platinum treatment appears to be a function of interacting processes (the accumulation and repair of damage) rather than of repair alone.

REFERENCES

1. Proceedings of the Third International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy. Oct. 18-20, 1976, Dallas, Texas. J. Clinical Hematology and Oncology Vol. 7, 1977.
2. Proceedings of the National Cancer Institute Conference on cis-platinum and Testicular Cancer. Cancer Treatment Reports Vol. 63, 1979.
3. J.J. Roberts and H.N.A. Fraval, The interaction of antitumour platinum compounds with cellular DNA in cultured cells and animal tissues : relationship to DNA cellular repair processes. Biochimie, 60 (1978) 869.
4. H.N.A. Fraval, C.J. Rawlings, and J.J. Roberts, Increased sensitivity of UV-repair-deficient human cells to DNA bound platinum products which unlike thymine dimers are not recognized by an endo-nuclease extracted from *Micrococcus Luteus*. Mutation Res. 51 (1978) 121.
5. A.G. Levis, X irradiation sensitivity of nitrogen mustard-resistant mammalian cells *in vitro*. Nature, 198 (1963) 498.
6. M.M. Elkind and G.F. Whitmore, The Radiobiology of Cultured Mammalian Cells. Gordon & Breach, New York, 1967, p. 41.
7. B. Drewinko and J.A. Gottlieb, Action of cis-dichlorodiammine platinum (II) (NSC119875) at the cellular level. Cancer Chemotherapy Reports, 59 (1975) 665.
8. H.W. Van den Berg and J.J. Roberts. Post-replication repair of DNA in Chinese hamster cells treated with cis platinum (II) diamine-dichloride. Enhancement of toxicity and chromosome damage by caffeine. Mutation Res. 33 (1975) 665.
9. H.W. Van den Berg and J.J. Roberts, Inhibition by caffeine of post-replication repair in Chinese hamster cells treated with cis platinum (II) diamine-dechloride : The extent of platinum binding to template DNA in relationship to the size of low molecular weight nascent DNA. Chem.-Biol. Interact.

12 (1976) 375.

10. A.M. Rauth, Evidence for dark-reactivation of ultraviolet light damage in mouse L cells. *Radiation Res.* 31 (1967) 121.
11. J.E. Cleaver and G.H. Thomas, Single strand interruptions in DNA and the effect of caffeine in Chinese hamster cells irradiated with ultraviolet light. *Biochem. Biophys. Res. Commun.*, 36 (1969) 203.
12. I. Szumiel, Response of two strains of L5178Y cells to cis dichlorobis (cyclopentylamine) platinum (II). I. Cross-sensitivity to cis PAD and UV light. *Chem.-Biol. Interact.* 24 (1979) 51.
13. I. Szumiel, Response of two strains of L5178Y cells to cis-dichlorobis (cyclopentylamine) platinum (II). II. Differential effects of caffeine. *Chem.-Biol. Interact.* 24 (1979) 73.

C. Borek (in collaboration with J.E. Cleaver. Laboratory
of Radiobiology, University of California, San Francisco)

Introduction

Protease inhibitors (Umezawa, 1976) inhibit mutation expression in bacteria (Meyn et al., 1977), inhibit the tumor-promoting effect of phorbol esters in mice and potentiate or inhibit *in vitro* transformation in rodent cells (Borek et al., 1979; Kuroki and Drevon, 1979) or human cells (Borek, 1980).

We therefore investigated DNA damage and repair in human fibroblasts by a variety of standard techniques to ascertain whether antipain has a discernible, direct effect on DNA. Antipain was also incubated with rat-liver microsomal S9 mix to determine whether it could be activated by mechanisms often observed with carcinogens (Miller and Miller, 1977). It was hoped that these experiments would aid in determining the mechanism of antipain in modifying cell transformation and the existence in mammalian cells of mechanisms analogous to the prokaryotic recA inducible system.

Methods

Normal human skin fibroblasts were grown in Eagle's minimal essential medium with 15% fetal calf serum and antibiotics. Cells were exposed to various concentrations of antipain or leupeptin in serum-free medium and then returned to medium with serum. Other cultures were irradiated with 254 nm ultraviolet (UV) light at an incident dose rate of $1.3 \text{ J/m}^2/\text{sec}$, or exposed only to serum-free medium for 30 min as controls. In some experiments, cells were exposed to antipain in medium containing serum or in medium containing 10% S9 mix (Ames et al., 1975; Miller and Miller, 1977). As controls, cells were also exposed for 30 min

to hydroxyurea (2 mM) or benzpyrene (30 µg/ml) with S9 mix.

DNA replication was determined by pulse-labeling cells for 15 min with 10 µCi/ml (^3H) thymidine (^3H dThd) (spec. act., 60 Ci/mmmole) at various times after exposure and determining ^3H incorporated into DNA (Painter, 1977). DNA damage was determined by assaying for single-strand breaks in alkaline sucrose gradients (5-20%, sedimented at 25,000 rev/min for 4 h) as described previously (Cleaver, 1975). DNA repair was assayed by three methods: incorporation of 10 µCi/ml (^3H)dThd (60 Ci/mmmole) for 2 h followed by autoradiographic analysis for unscheduled DNA synthesis (Cleaver, 1975); incorporation of 20 µCi/ml (^3H)BrdUrd (10 µM) for 3 h followed by isolation of DNA and analysis for repair replication in isopycnic gradients (Cleaver, 1975); or the accumulation of excision-repair breaks after exposure to cytosine arabinoside (Hiss and Preston, 1977; Dunn and Regan, 1979; Hashem et al., 1980).

Results

Inhibition of DNA synthesis. Measurement of DNA synthesis at various times after exposure of cells to various agents has recently been developed as a simple method that distinguishes between inhibitors of DNA synthesis that act by damaging DNA and those that act by less specific mechanisms (Painter, 1977, 1978). After exposure to DNA-damaging agents such as UV light or activated benzpyrene, DNA synthesis continued to decline because of the persistence of damaged sites in DNA. After exposure to inhibitors that do not damage DNA, such as hydroxyurea, cells rapidly recovered their DNA synthesis capacity (Fig. RB-11-1). Exposure to S9 mix alone had a similar effect, with rapid recovery of DNA synthesis upon its removal (Fig. RB-11-2). Antipain alone was completely without effect (Fig. RB-11-1), but the presence of serum or S9 mix caused a transient inhibition of DNA synthesis followed by a rapid recovery (Fig. RB-11-2). This effect resembles that of

Figure 1: Relative rate of DNA synthesis in human fibroblasts exposed to 1.7 mM antipain for 30 min. (closed square), 13 J/m² of 254 nm UV light (open circle) or 2 mM hydroxyurea for 30 min (open square) and pulse-labeled for 15 min with 10 μ Ci/ml (³H)dThd (60 Ci/mmol) at various times after exposure.

Figure 2: Relative rate of DNA synthesis in human fibroblasts exposed for 30 min to S9 mix (closed circle), 1.7 mM antipain plus 10% serum (closed triangle), 1.7 mM antipain plus S9 mix (open triangle) or 30 μ g/ml benzpyrene plus S9 mix (inverted triangle) and pulse-labeled for 15 min with 10 μ Ci/ml (³H)dThd (60 Ci/mmol) at various times after exposure.

hydroxyurea and is quite unlike that which is typical of DNA-damaging agents, e.g., UV light (Fig. RB-11-1) and activated benzpyrene (Fig. RB-11-2).

Single-strand break production. Cells prelabeled with 0.01 $\mu\text{Ci/ml}$ (^{14}C)dThd (64 mCi/mmol) were exposed for 30 min to 1.7 mM antipain or 2.5 mM leupeptin and immediately analyzed on alkaline sucrose gradients (Fig. RB-11-3). Single-strand sizes from control cells were 1 to 2×10^8 daltons, and no breakage by protease inhibitors was detected on gradients of the treated cells.

Accumulation of excision-repair breaks in the presence of cytosine arabinoside. The excision and repair of DNA damage can be blocked by incubation in cytosine arabinoside, which results in the accumulation of breaks at each excision repair site (Hiss and Preston, 1977; Dunn and Regan, 1979; Hashem et al., 1980). Growth for 4 to 5 h in cytosine arabinosine after 1.3 to 13 J/m^2 UV light resulted in large reductions in single-strand size (Figs. RB-11-4 and RB-11-5). The reduction in molecular weight following 13 J/m^2 UV light corresponded to the accumulation of approximately 6 to 7 breaks per 10^8 daltons in 5 h (Hashem et al., 1980). No accumulation of breaks was detected in the presence of cytosine arabinoside 4-5 h after exposure to antipain or leupeptin, with or without S9 activation (Figures RB-11-4 and RB-11-5).

Repair replication. Alkaline isopycnic gradient centrifugation of control and UV-irradiated fibroblasts labeled with (^3H)BrdUrd produced heavy- and light-density ^3H -labeled DNA, respectively (Fig. RB-11-6). In control cultures the heavy-density DNA corresponds to DNA strands labeled extensively with long stretches of BrdUrd. After UV irradiation, semi-conservative DNA synthesis was inhibited and the incorporation of (^3H)BrdUrd into small repair patches produced ^3H -labeled light-density DNA (Fig. RB-11-6). After exposure to antipain (0.7 mM) or leupeptin (1.1 mM), however, only heavy ^3H -labeled DNA strands were obtained

Figure 3: Alkaline sucrose gradient profiles of $(^{14}\text{C})\text{dThd}$ -labeled human fibroblasts exposed for 30 min to 1.7 mM antipain (square) or 2.5 mM leupeptin (triangle) and analyzed immediately. Sedimentation from right to left at 25,000 rev/min for 4 h at 18°C. Arrow denotes peak fraction of unexposed control cells. Actual counts are displayed without normalization.

Figure 4: Alkaline sucrose gradient profiles of human fibroblasts exposed for 30 min to 1.7 mM antipain (closed square) or 2 mM leupeptin (closed triangle), to 13 J/m² of 254 nm UV light (closed circle), or unexposed (open circle), and then grown for 5 h in 20 M cytosine arabinoside and 2 mM hydroxyurea. Sedimentation from right to left at 25,000 rev/min for 4 h at 18°C. Actual counts are displayed without normalization.

Figure 5: Alkaline sucrose gradient profiles of human fibroblasts exposed for 30 min to 0.8 mM antipain plus S9 mix (●) or 0.2 mM antipain plus S9 mix (square), to 1.3 J/m² of 254 nm UV light (Δ), or to 5.2 J/m² UV light (▲) and grown for 4 h in 20 μM cytosine arabinoside and 2 mM hydroxyurea. (---) Control values. Sedimentation is from right to left. For technical reasons it was necessary to sediment at 10,000 rev/min for 16 h at 40°C, rather than at 25,000 rev/min, thus causing control peaks to be higher in the gradient and more symmetrical than in Fig. 4. Data have been normalized to the same peak radioactivity.

Figure 6: Alkaline isopycnic gradient profiles of human fibroblasts exposed for 30 min to 0.7 mM antipain (square) or 1.1 mM leupeptin (Δ), to 13 J/m² of 254 nm UV light (●), and then labeled for 3 h in 20 μCi/ml (³H)BrdUrd (10 μM) and FdUrd (1 μM), and the DNA was isolated. Centrifugation was at 42,000 rev/min for 48 h, density increases from left to right, absorbance at 260 nm.

(Fig. RB-11-6) and no repair replication was detected.

Unscheduled DNA synthesis. Autoradiographs of control cultures gave a low frequency of heavily ^3H -labeled S phase cells, and the remainder of the cell population (G_1 and G_2 phases) were unlabeled. After UV irradiation the G_1 and G_2 cells incorporated a low level of (^3H)dThd during repair of the UV damage. The average grain number of lightly labeled cells in autoradiographs of cells exposed to UV, antipain or leupeptin indicated repair of UV-damaged cells only (Table RB-11-1). Exposure of cells to antipain or leupeptin after UV irradiation had no effect on the amount of UV-induced unscheduled synthesis (Table RB-11-1).

DNA replication after UV or X-irradiation in the presence of antipain. Most DNA-damaging agents characteristically inhibit either the initiation of DNA replication, DNA chain growth or both. X-rays inhibit initiation by causing strand breaks (Painter and Young, 1976; Walters and Hildebrand, 1975). UV light inhibits initiation at low doses, presumably by means of excision-repair breaks (Park and Cleaver, 1979; Cleaver, 1978a; Kaufmann et al., in press), and inhibits chain elongation at higher doses by means of pyrimidine dimers (Lehmann, 1979; Cleaver et al., 1979; Kaufmann et al., in press). Because antipain slightly depressed the rate of DNA synthesis (Fig. RB-11-2), we determined the size distribution of nascent DNA chains after 1 h of exposure to a high dose of antipain (1.7 mM) to see whether there were any characteristic alterations.

A 10-min pulse of (^3H)dThd gave a broad profile of labeled DNA with a peak in the region of 40S corresponding to recently initiated replicons and a shoulder up to 100S representing extended and linked clusters of replicons (Fig. RB-11-7, top). After 1.2 krad of x-rays the peak at 40S was eliminated, indicating a reduction in the number of replicons initiating during the labeling period (Fig. RB-11-7, center). After 1.3 J/m^2 UV light a similar reduction in

Table RB-11-1

Unscheduled DNA synthesis in human fibroblasts exposed to 13 J/m² UV light and/or various concentrations of antipain or leupeptin and labeled for 1 hr in 10 µCi/ml (³H)dThd (60 Ci/mmol)^a

UV (J/m ²)	Antipain, mM					Leupeptin, mM
	0	0.02	0.17	0.9	1.7	2.0
0	0	0.2	0.2	0.2	0.6	0.1
13	28.8 ± 1.1 ^b	27.9	28.3	28.0	27.7	28.3

^a Autoradiographs were prepared with Kodak NTB liquid emulsion and exposed for four days before developing D19 developer; average grain numbers were determined by counting 30 lightly labeled cells per slide. Background of 0.9 grain/cell on control slides was subtracted.

^b Average grain numbers from five separate slides are combined and expressed as mean ± standard error. All other determinations are based on single slides.

Figure 7: Alkaline sucrose gradients of human fibroblasts irradiated with 0 or 1.2 krad of x-rays (50 kVp), 1.3 or 5.2 J/m² UV light, grown for 1 h in fresh medium plus serum with or without 1.7 mM antipain, labeled for 10 min in the same medium with 10 µCi/ml (³H)Thd (60 Ci/mmol) and analyzed immediately. Top, control. Center, 1.2 krad of x-rays. Bottom, 1.3 (o, ●) or 5.2 (closed square) J/m² UV light. Closed symbols, without antipain; open symbols, with antipain. Centrifugation from right to left, 25,000 rev/min for 4 h, calibration with simian virus 40 and lambda viral genomes.

initiation was observed, but there was also a reduction at higher molecular weights corresponding to an effect of blocking chain growth and linkage of replicon clusters (Fig. 11-7, bottom). After 5.2 J/m^2 a further reduction in DNA replication was observed and there was an accumulation of short fragments below 40S representing the blocking of chain growth by pyrimidine dimers. The effects of x-ray-induced strand breaks and UV-induced pyrimidine dimers on DNA replication were not influenced by the presence of antipain for 1 h between irradiation and labeling (Fig. RB-11-7, center and bottom). Antipain alone had a slight effect on the amount of label found in DNA molecules in the size range around 40S. This effect, if caused by a mechanism similar to that involved with x-rays, could suggest a small number of direct strand breaks similar to those produced by 100-200 rads of x-rays. However, this effect was observed at a high dose (1.7 mM) of antipain, and its effects on cell transformation have been observed at 100- to 1000-fold lower concentrations (Borek et al., 1979; Borek, 1980). It is unlikely, therefore, that this very small effect has any importance in the biological effects of antipain on cell transformation.

Discussion

Several recent studies have indicated that protease inhibitors can potentiate transformation of rodent and human cell cultures by radiation (Borek et al., 1979; Borek, 1980). It was reasonable, therefore, to investigate whether some of the effects of antipain and leupeptin on cell transformation could be attributed to a direct production of DNA damage or inhibition of DNA repair. The aldehyde group present in one form of antipain is a potential reactive group that could interact with DNA (Umezawa, 1976). A preliminary report has suggested that antipain is more toxic to excision-repair-deficient xeroderma pigmentosum cells than to normal human cells (Takeda and Ishizaki, 1978); this could be explained if

antipain produced DNA damage which could not be repaired in xeroderma pigmentosum cells. If this were proved correct, then the mechanism by which antipain potentiates radiation-induced transformation would be similar to that which underlies the synergism often observed between two different carcinogens (DiPaolo et al., 1971, 1974, 1976).

We were unable, however, to obtain any evidence for the production of DNA damage, including DNA strand breaks, with or without metabolic activation, or for the involvement of excision repair, or for the inhibition of UV excision-repair by concentrations of antipain up to 2 mM. We consider it unlikely, therefore, that any direct production of DNA damage or inhibition of repair is involved in the effects of protease inhibitors in mammalian cell transformation.

Antipain has been found to inhibit proteases involved in the recA-mediated SOS repair functions in E. coli (Meyn et al., 1977; Troll et al., 1978), but its role in mammalian cell transformation cannot be taken as evidence for any analogous system in mammalian cells. There is no clear evidence that such an inducible error-prone system has major importance for mammalian cells (Cleaver, 1978a,b; Chang et al., 1978), and alternative arguments have been presented in detail elsewhere (Cleaver, 1978a). Although there is evidence for an inducible error-prone system unique to simian virus 40-infected cells, this may be a special case because of the unusually high reversion frequencies seen in that system (Sarasin and Benoit, 1980; Cleaver and Weil, 1975), and not indicative of processes that operate in uninfected cells.

The effect of antipain on cell transformation must therefore be explained by mechanisms at least one step removed from direct involvement with DNA damage and repair. It may be effective, for example, at the cell surface and modulate cell communication and expression of transformation. In this connection, one

important question not yet fully addressed is the extent to which antipain actually penetrates cells and interacts with cytoplasmic and nuclear components. The slight inhibition of DNA synthesis by antipain (Fig. RB-11-2) in the presence of serum and S9 mix could be due to increased absorption by the cell.

We intend to continue these investigations to further delineate those cellular systems that are or are not involved in the observed potentiating effects of antipain on cell transformation, so that we can understand the mechanisms of transformation in various rodent and human cell systems.

Acknowledgements

Antipain and leupeptin were donated by Dr. W. Troll through the U.S.-Japan Cooperative Program. We are grateful to Ms. R. Howard for preparation of the S9 mixture.

References

- Ames, B.N., J. McCann and E. Yamasaki, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, *Mutation Res.* 31:347-364, (1975).
- Borek, C., X-ray-induced *in vitro* neoplastic transformation of human diploid cells, *Nature* 283:776-778, (1980).
- Borek, C., R. Miller, C. Pain and W. Troll, Conditions for inhibiting and enhancing effects of the protease inhibitor antipain on x-ray-induced neoplastic transformation in hamster and mouse cells, *Proc. Natl. Acad. Sci. (U.S.A.)* 76:1800-1803, (1979).
- Chang, C.-C., S.M. d'Ambrosio, R. Schultz, J.E. Trosko and R.B. Setlow, Modification of UV-induced mutation frequencies in Chinese hamster cells by dose fractionation, cycloheximide and caffeine treatments, *Mutation Res.* 52:231-245, (1978).
- Cleaver, J.E., Methods for studying repair of DNA damaged by physical and chemical carcinogens, in: H. Busch (Ed.), *Methods in Cancer Research*, Vol. 11, Academic Press, New York, 1975, pp. 123-165.
- Cleaver, J.E., DNA repair and its coupling to DNA replication in eukaryotic cells, *Biochim. Biophys. Acta*, 516:489-516, (1978a).

- Cleaver, J.E., Absence of interaction between x-rays and UV light in inducing ouabain- and thioguanine-resistant mutants in Chinese hamster cells. *Mutation Res.* 52:247-253, (1978b).
- Cleaver, J.E. and S. Weil, UV-induced reversion of a temperature-sensitive late mutant of simian virus 40 to a wild-type phenotype, *J. Virol.* 16:214-216, (1975).
- Cleaver, J.E., G.H. Thomas and S.D. Park, Xeroderma pigmentosum variants have a slow recovery of DNA synthesis after irradiation with ultraviolet light, *Biochim. Biophys. Acta*, 564:122-131, (1979).
- DiPaolo, J.A., P.J. Donovan and B.C. Casto, Enhancement by alkylating agents of chemical carcinogen transformation of hamster cells in culture. *Chem.-Biol. Interact.* (:351-364, (1974).
- DiPaolo, J.A., P.J. Donovan and R.L. Nelson, X-irradiation enhancement of transformation by benzo(a)pyrene in hamster embryo cells, *Proc. Natl. Acad. Sci. (U.S.A.)*, 68:1734-1737, (1971).
- DiPaolo, J.A., P.J. Donovan and N.C. Popescu, Kinetics of Syrian hamster cells during x-irradiation enhancement of transformation *in vitro* by chemical carcinogens, *Radiat. Res.* 66:310-325, (1976).
- Dunn, W.C. and J.D. Regan, Inhibition of DNA excision repair in human cells by arabinofuranosyl cytosine: Effect on normal and xeroderma pigmentosum cells, *Mol. Pharmacol.* 15:367-374 (1979).
- Hashem, N., D. Bootsma, W. Keijzer, A. Greene, L. Coriell, G. Thomas and J.E. Cleaver, Clinical characteristics, DNA repair, and complementation groups in xeroderma pigmentosum patients from Egypt, *Cancer Res.* 40:13-18, (1980).
- Hiss, E.A. and R.J. Preston, The effect of cytosine arabinoside on the frequency of single-strand breaks in DNA of mammalian cells following irradiation or chemical treatment, *Biochim. Biophys. Acta* 478:1-8, (1977).
- Kaufmann, W.K., J.E. Cleaver and R.B. Painter, Ultraviolet radiation inhibits replicon initiation in S phase human cells, *Biochim. Biophys. Acta*, in press.
- Kuroki, T. and C. Drevon, Inhibition of chemical transformation in C3H 10T $\frac{1}{2}$ cells by protease inhibitors, *Cancer Res.* 39:2755-2761 (1979).
- Lehmann, A.R., The relationship between pyrimidine dimers and replicating DNA in UV-irradiated human fibroblasts. *Nucleic Acids Res.* 7:1901-1912, (1979).
- Meyn, M.S., T. Rossman and W. Troll, A protease inhibitor blocks SOS functions in Escherichia coli: Antipain prevents λ repressor inactivation, ultraviolet mutagenesis, and filamentous growth, *Proc. Natl. Acad. Sci. (U.S.A.)* 74:1152-1156, (1977).

- Miller, J.A. and E.C. Miller, Ultimate chemical carcinogens as reactive mutagenic electrophiles, in: H.H. Hiatt, J.D. Watson and J.A. Winsten (Eds.), *Origins of Human Cancer*, Book B, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977, pp. 605-627.
- Painter, R.B., Rapid test to detect agents that damage human DNA, *Nature* 265:650-650, (1977).
- Painter, R.B., DNA synthesis inhibition in HeLa cells as a simple test for agents that damage human DNA, *J. Environ. Path. Toxicol.* 2:65-78, (1978).
- Painter, R.B. and B.R. Young, Formation of nascent DNA molecules during inhibition of replicon initiation in mammalian cells, *Biochim. Biophys. Acta* 418:146-153, (1976).
- Park, S.D. and J.E. Cleaver, Recovery of DNA synthesis after ultraviolet irradiation of xeroderma pigmentosum cells depends on excision repair and is blocked by caffeine, *Nucleic Acids Res.* 6:1151-1159, (1979).
- Sarasin, A. and A. Benoit, Induction of an error-prone mode of DNA repair in UV-irradiated monkey kidney cells, *Mutation Res.* 70:71-81, (1980).
- Takeda, H. and K. Ishizaki, High sensitivity of xeroderma pigmentosum cells to antipain, a protease inhibitor (abstract), *Proceedings of the Japanese Cancer Association* 11:13, (1978).
- Troll, W., S.M. Meyn and T.G. Rossman in: T.J. Slaga, A. Swak and R.K. Boutwell (Eds.), *Mechanisms of Tumor Promotion and Carcinogenesis*, Raven Press, New York, 1978, pp. 301-311.
- Umezawa, H., Structures and activities of protease inhibitors of microbial origin, *Meth. Enzymol.* 45:678-695, (1976).
- Walters, R.A. and C.E. Hildebrand, Evidence that x-irradiation inhibits DNA replicon initiation in Chinese hamster cells, *Biochem. Biophys. Res. Commun.* 65:265-271, (1975).

RB-12 Effects of long contact times of misonidazole on attached Chinese hamster V-79 cells

C.R. Geard and M.H. Rutledge-Freeman

Introduction

Hypoxic cells in tumors have frequently been proposed as the limiting factor for radio-curability of some human tumors (6). The basis for sensitization of hypoxic mammalian tumor cells to radiation damage have been presented by Adams (1). Briefly, radiosensitizers should be nontoxic to normal tissues, to have minimal sensitizing action on oxygenated cells, to penetrate rapidly to non-vascularized regions of a tumor, and to be relatively resistant to metabolic breakdown. Some of the above criteria have been met in the development of misonidazole, a 2-nitroimidazole, which is undergoing clinical trials as an adjunct to radiation therapy (5, 12, 15). Misonidazole appears to act relatively selectively on hypoxic cells as both a radiosensitizer and cytotoxic agent with limited killing effects on aerated cells except at high concentrations and/or prolonged contact times (7, 9). Olive (10) has, however, shown that short contact times (to 4 hours) effect DNA synthesis of aerated mouse L cells at high concentrations while more significantly Miller and Hall (8) showed the induction on oncogenic transformation in aerated mouse C3H10T $\frac{1}{2}$ cells after long drug contact times (3 or 6 days) at low concentrations. This study presents data on sister chromatid exchanges (S.C.E.'s), chromosomal aberrations, cell cycle effects, and cell survival after incubating attached Chinese hamster V-79 cells with misonidazole for 24, 48, and 72 hours. Increases in S.C.E.'s were comparatively small and chromosomal aberrations few, even when 90% of cells failed to survive, while progression through the cell cycle was slowed both by increasing drug concentrations and contact times.

Materials and Methods

Chinese hamster V-79 cells were grown at 37.5°C in Falcon plastic flasks in Medium F10 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum, antibiotics, and L-glutamine.

Misonidazole (donated by Hoffman-La Roche, Inc., Nutley, NJ) was added to aerated cells growing in log phase to concentrations of 1, 2, and 5 mM and cultures returned to the incubator. Bromodeoxyuridine (BrdU) at 5 μ M was added at 4, 28, and 52 hours after the addition of the misonidazole. In each case, 16 hours later (time for chromosomes to have incorporated BrdU over two replication cycles) colcemid (3 μ M) was added to the cultures 4 hours prior to harvest. Misonidazole was in contact with cells for 24, 48, and 72 hours and chromosomal effects were examined in cells replicating and then proceeding into mitosis over the final 20 hours. During the whole incubation and cell preparation period, cultures were maintained in total darkness or subdued light. Cells were collected by trypsinisation, made hypotonic in 0.075 M KCl, and fixed in cold 3:1 methanol, acetic acid. The fixative was changed twice, and the fixed cells dropped on wet slides and air dried.

Chromatid differentiation for S.C.E. analysis was carried out by incubating slides in 0.3 M Na_2HPO_4 at pH 10.4 containing 2% Gurr's R66 Giemsa stain for 12-20 mins. (2). This one step procedure results in a reverse chromatid differentiation to the Hoechst-Giemsa differentiation procedure and can be used at once. Mitoses after one round of replication in BrdU show both chromatids uniformly stained, after 2 rounds one chromatid equivalent per chromosome is deeply stained and the other lightly stained while after 3 rounds some chromosomal segments are deeply, uniformly stained in both chromatids while others are differentially stained. S.C.E.'s were scored in

1000 second division chromosomes from each treatment, and also in chromosomes in third division cells which had the cytological appearance of the 2nd division chromosomes. These chromosomes had proceeded through two cellular division without an S.C.E. and thus, could demonstrate S.C.E.'s from the immediately prior cycle only. In this instance, 200 chromosomes were scored. The frequency of mitotic cells in the first, second or third divisions was assessed in 200 cell samples from each treatment, while mitotic indices were determined from 2,000 cell samples.

Scoring of chromosomal aberrations was carried out on slides stained with 2% aceto-orcein, since Giemsa staining is inadequate for stringent aberration analysis. 50 cells were scored from each treatment.

For assaying cell survival (clonogenicity) medium was changed after the appropriate misonidazole contact time and cultures incubated for 6 to 8 days before fixing, staining, and colony counting. In this instance, the effects of drug contact alone was compared with drug plus BrdU for the final 20 hours of treatment. By comparison with controls, the fraction of cells surviving each treatment schedule was determined.

Results

Cell survival

Figure 1 shows survival curves for cells exposed to misonidazole for 24, 48, and 72 hours in the presence of absence or 5 μ M BrdU for the final 20 hours of drug treatment. At 1 and 2 mM misonidazole there are comparatively small differences from control, however, 5 mM shows a substantial increase in effect as contact time increases and also shows a consistent enhanced effect in the presence of BrdU.

Figure 1. Proportion of surviving Chinese hamster V-79 cells after incubation with misonidazole for different times in the presence or absence of bromodeoxyuridine for the final 20 hours of treatment.

Cell cycle effects

Mitotic indices were assessed from colcemid accumulations of mitoses over the last 4 hours of treatment. The proportions of mitoses relative to control are shown in Figure 2. Control mitotic indices were 19.8, 23.2 and 23.3 percent respectively for the 24, 48, and 72 hour experiments. Here again, a 5 mM concentration of misonidazole produces a substantial reduction in mitoses, particularly after 48 and 72 hours of drug contact.

It thus appears that a smaller fraction of cells will move through the cell cycle as drug concentration and contact time increases.

The frequency of mitoses in different cell cycles indicates the rate of movement of cells through the cell cycle in the presence of drug. As can be seen in Figure 3, after incubation in BrdU the three control cultures allow a substantial fraction of cells to cycle through three cellular divisions in 20 hours. This fraction of cells in the third division decreases as drug concentration increases. In particular for 5 mM misonidazole, all cycling cells have only moved through 2 divisions for 24 hours contact, while 50% of cells move through 2 divisions for 48 hours contact, and this drops to less than 10% after 72 hours contact. Thus, increasing drug concentrations and contact times progressively slow the movement of cycling cells through the cell cycle.

Chromosomal effects

The frequency of S.C.E.'s in chromosomes after 2 cycles of BrdU incorporation is shown in Figure 4, along with frequencies of S.C.E.'s in chromosomes from third division cells that had arrived at mitosis with the cytological appearance of second division chromosomes. As is obvious from Figure 3, the 5 mM drug concentration did not allow any cells to progress through 3 replication cycles, so data are only presented for 0, 1, and 2 mM drug.

There appears to be an effect of misonidazole in inducing S.C.E.'s in

Figure 2. Proportion of mitoses relative to control found after colcemid accumulation for the final 4 hours of the time Chinese hamster V-79 cells were incubated with misonidazole.

Figure 3. Percentages of mitoses in the 1st, 2nd, or 3rd divisions after bromodeoxyuridine was made available to Chinese hamster V-79 cells 20 hours prior to fixation.

Figure 4. Sister chromatid exchanges per chromosome from 2nd and 3rd division mitoses and chromosome aberrations per cell from Chinese hamster V-79 cells after bromodeoxyuridine was made available for the final 20 hours of misonidazole treatment. Standard errors of the mean are indicated.

2nd division chromosomes though this is always less than a two-fold increase, and whether there is a consistent dose-response relation or a plateauing effect in exchange induction cannot be determined.

If a clastogenic agent induces an equivalent number of S.C.E.'s in each cell cycle then the level of exchanges seen in 3rd division chromosomes without prior S.C.E.'s should be half the level of that seen in 2nd division chromosomes (16). For control cells the levels are approximately halved as might be expected, however, in most instances the levels from the drug treated cells are not different from the control and at 72 hours particularly, much less than half that found in 2nd division. This may reflect a selective effect in that cells cycling faster have less time available to express S.C.E.'s or perhaps the efficiency of the drug as an S.C.E. inducer is declining with incubation time.

Chromosome aberrations per cell are also shown on the same scale as S.C.E.'s per chromosome. It is apparent that the level of induced chromosomal aberrations is low. Of those aberrations scored, 64% were chromatid deletions, 9% isochromatid deletions, and 16% chromatid exchanges. The remainder comprised a few chromatid intrachanges and dicentrics. Over 90% of aberrations were chromatid-type aberrations which were most probably induced in the cell cycle immediately prior to the mitosis at which they were scored. The majority of cells scored after 5 mM misonidazole treatment only proceeded through one replication cycle during the final 20 hours of incubation in drug and in no instance is the level of aberrations significantly different from that in the controls. Overall, there is an enhanced level of drug induced aberrations, but this bears no relation to the level of cell killing induced at each drug concentration and contact time.

Discussion

These results demonstrate that, at high concentrations and long contact times, misonidazole which is a very efficient agent against hypoxic cells also kills aerated cells. This killing effect is enhanced if BrdU is incorporated into chromosomes providing suggestive evidence that perturbations at the DNA level are, at least in part, responsible for cell death. This finding differs from that of Stratford and Gray (14) who (also with Chinese hamster V-79 cells) did not find an enhanced misonidazole cytotoxicity in the presence of BrdU. In contrast to this study, they worked with spinner cultures and examined one misonidazole concentration (2 mM) over increasing incubation times.

The finding of comparatively few mitoses, and these only cycling slowly at high drug levels and contact times, in conjunction with the finding of few chromosomal aberrations suggests that the majority of non-surviving cells die in interphase. Therefore, most non-survivors do not suffer from induced chromosomal aberrations and post-mitotic cell death.

These conclusions, based on long drug contact times on aerated cells, are compatible with those proposed previously to explain the cytotoxic action of misonidazole on hypoxic cells (4). Short contact times (to 4 hours) on hypoxic cells resulted in considerably lowered cell survival, the almost complete cessation of cellular progression, and low though disproportionately distributed cellular levels of chromosomal aberrations.

In this study, the small number of aberrations seen were concentrated in cells treated with low drug levels; the majority were chromatid-type and there were no cells with high levels of aberrations.

The levels of S.C.E.'s found are comparatively slight when contrasted with those induced by common chemotherapeutic agents (11, 13, 16). It must

be concluded that, using S.C.E.'s as an indicator of mutagenicity-carcinogenicity, misonidazole does not classify as an efficient mutagen-carcinogen (11).

Whilst this drug is capable of cytotoxic effects, of delaying cell progression, and possibly, of inducing low levels of S.C.E.'s and chromosomal aberrations, these are all at high dose levels. Therefore, though human usage will result in normal aerated cells being exposed to drug for quite long periods, this will be at doses less than 1 mM (5). On these grounds, these data would imply that misonidazole is a comparatively safe agent, particularly when its effects are contrasted with those of other chemotherapeutic agents (3).

REFERENCES

1. Adams, G.E. Chemical radiosensitization of hypoxic cells. Br. Med. Bull 29:48-53 (1973).
2. Alves, P. and Jonasson, J. New staining method for the detection of sister chromatid exchanges in BrdU labelled chromosomes. J. Cell Sci. 32:185-195 (1978).
3. Benedict, W.F., Banerjee, A., Gardner, A., and Jones, P.A. Induction of morphological transformation in mouse C3H10T $\frac{1}{2}$ clone 8 cells and chromosomal damage in hamster A (T1) C1-3 cells by cancer chemotherapeutic agents. Cancer Res. 37:2202-2208 (1977).
4. Geard, C.R. Povlas, S.F., Astor, M.B., and Hall, E.J. Cytological effects of 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol (misonidazole) on hypoxic mammalian cells *in vitro*. Cancer Res. 38:644-649 (1978).
5. Gray, A.J., Dische, S., Adams, G.E., Flockhart, I.R., and Foster, J.L. Clinical testing of the radiosensitizer Ro-07-0582. 1. Dose tolerance, serum and tumour concentrations. Clin. Radiol. 27:151-157 (1976).

6. Hall, E.J. Radiobiology for the Radiologist, 2nd Edition. Harper and Row, New York, 1978.
7. Hall, E.J. and Biaglow, J. Ro-07-0582 as a radiosensitiser and cytotoxic agent. Intern J. Radiation Oncol. Biol. Phys. 2:521-530 (1977).
8. Miller, R.C. and Hall, E.J. Oncogenic transformation *in vitro* by the hypoxic cell sensitizer misonidazole. Br. J. Cancer 38:411-417 (1978).
9. Moore, B.A., Palcic, B. and Skarsgard, L.D. Radiosensitising and toxic effects of the 2-nitromidazole Ro-07-0582 in hypoxic mammalian cells. Radiation Res. 67:459-473 (1976).
10. Oliver, P.L. Inhibition of DNA synthesis by nitroheterocycles. I. Correlation with half-wave reduction potential. Brit. J. Cancer 40:89-93 (1979).
11. Perry, P. and Evans, H.J. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258:121-125 (1975).
12. Phillips, T.L., Wasserman, T.H., Johnson, R.J., Gomer, C.J., Lawrence, G.A., Levine, M.L., Sadee, W., Penta, J.S. and Rubin, D.J. The hypoxic cell sensitiser programme in the United States. Br. J. Cancer 37:276-280 (1978).
13. Popescu, N.C., Turnbull, D., DiPaolo, J.A. Sister chromatid exchange and chromosome aberration analysis with the use of several carcinogens and non-carcinogens. J. Natl. Canc. Inst. 59:289-293 (1977).
14. Stratford, I.J. and Gray, P. Some factors affecting the specific toxicity of misonidazole towards hypoxic mammalian cells. Brit. J. Cancer 37:129-131 (1978).

15. Thomlison, R.Y., Dische, S., Gray, A.J. and Errington, I.M. Clinical studies of the radiosensitizer Ro-07-0582. III. Response of tumours. Clin. Radiol. 27:167-174 (1976).
16. Wolf, S. Sister chromatid exchange. Ann. Rev. Genet. 11:183-201 (1977).

Charles R. Geard, Louis E. Schwartz, and Melanie Rutledge-Freeman

Introduction

The ability of misonidazole to selectively radiosensitize hypoxic mammalian cells is now well established. It has also been demonstrated that the nitroimidazoles are also more toxic to hypoxic cells than to oxic cells. The mechanism of action of this cytotoxicity is poorly understood, and may be independent of the mechanisms of radiosensitization. This cytotoxic effect may be significant since hypoxic cells may also be resistant to chemotherapeutic agents in current usage.

If misonidazole is to be used widely in patients with a relatively good prognosis, the potential for long term effects on normal cells needs to be established. The studies reported here were designed to assess potentially hazardous effects and to address the mechanisms responsible for the cytotoxic effects of the drug. Clonogenicity, progression through the cell cycle, chromosomal aberrations, sister chromatid exchanges and oncogenic transformations have been scored in aerated mouse C3H/10T $\frac{1}{2}$ fibroblasts treated with misonidazole, and in aerated and hypoxic Chinese hamster V-79 cells.

Materials and Methods

a) Mouse C3H/10T $\frac{1}{2}$ fibroblasts

Cells between the 12th and 16th passages were used for assaying oncogenic transformation, while cells from the 12th to the 19th passage were used for assessing cell survival and chromosomal effects. Cells were grown at 37.5°C in Corning plastic petri dishes in Eagle's basal medium (BME), supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin.

Clonogenic ability of these cells was determined by exposing asynchronous

attached cells to misonidazole at concentrations between 1 and 6 mM for 6, 24, 48 and 72 hours. Giemsa stained colonies were counted on approximately 20 dishes per treatment (3 experiments) after a 2 week incubation period.

Mitotic indices and chromosomal aberrations were studied in cells exposed to misonidazole at a concentration of 1 to 6 mM to which colcemid (5 μ M) was added at four 2-hour intervals from 0 to 8 hours post-contact and for 4 hours at 20, 44 and 68 hours post-contact. Mitotic indices were assessed from samples of 1,000 cells while chromosomal observations were made in 50 or 100 near-tetraploid cells from each dose-time regimen.

Sister chromatid exchange (S.C.E.) frequencies were assessed following bromodeoxyuridine (BUdR) incorporation into chromosomes. Misonidazole (1, 3 or 6 mM) was added to attached asynchronous cells together with 3 μ M BUdR. Colcemid (5 μ M) was added 20, 28 or 44 hours later. Mitotic cell accumulation was allowed for 4 hours before fixation. Chromosomal spreads were prepared and slides stained with 2% Giemsa in 0.3 M Na_2HPO_4 adjusted to pH 10.4 - 10.8 with 1 M NaOH for chromatid differentiation. Second division chromosomes (1,000 scored) were found at 24 and 32 hours while cells collected at 48 hours contained primarily chromosomes which had gone through three replication rounds. Some of these chromosomes are visually similar to second division chromosomes in that they passed through two cycles without an exchange. Assessing SCE's in such chromosomes (200 scored per treatment) allows an estimate of exchange induction over one cycle only and provides a test of whether the efficacy of the compound under investigation is diminishing with time.

Oncogenic transformation *in vitro* was determined for misonidazole at a concentration of 1 mM, with exposure times of 1, 3 and 6 days. This concentration was chosen for intensive study since it represents the maximum clinically feasible dose. Transformed foci (Types II and III) were scored 5 to 6 weeks after the

initiation of the experiments.

b) Chinese hamster V-79 cells

Cells were grown at 37.5°C in Falcon plastic flasks in Medium F10 supplemented with 10% fetal calf serum, antibiotics and L-glutamine. Cell suspensions were dispensed, with or without misonidazole (1, 2 and 5 mM), into glass ampules; 2×10^6 cells in 1 ml for hypoxic cells, and 10^5 cells in 1 ml for aerated cells. Ampules were flushed with high purity nitrogen plus 5% CO₂ (hypoxic) or air plus 5% CO₂ (oxic) before heat sealing and agitating in a 37.5°C water bath for 4 hours (hypoxic) or maintained at room temperature for the same period (oxic) cells.

Clonogenic ability was examined for cells which were treated with misonidazole alone or misonidazole plus 5 µM BUdR. Incorporation of the analogue into DNA was allowed for 16 hours (two replication cycles) before cells were sealed in suspension in the ampules. Replated cells were incubated for 8 days before they were fixed, stained and colonies counted.

Progression through the cell cycle, the frequency of chromosomal aberrations per cell and the frequency of SCE's were assessed in cells at metaphase that were sequentially accumulated with colcemid (5 µM) over eight 2 hour periods (to 16 hours) after treatment. Mitotic indices were scored on 1,000 cell samples, chromosomal aberrations in 50 cell samples and SCE's in 1,000 chromosome samples for each dose-time regimen, and results were combined for the total 16 hour accumulation period.

Results and Discussion

Mouse C3H/10T½ fibroblasts

The survival curves shown in Fig. 1 clearly demonstrate that increasing drug concentration and increasing drug contact time enhance lethality in these attached aerated cells. Given the known capacity of chromosome aberrations to result in cell lethality, Table 1 presents data on the frequency of mitoses and the frequency of chromosomal aberrations for increasing misonidazole contact times. The frequency

Figure 1: The proportion of surviving mouse C3H 10T½ cells versus misonidazole concentration (1 to 6 mM) for contact times of 6 (circles), 4 (squares), 48 (triangles) and 72 hours (diamonds). Standard errors are indicated.

Figure 2: The frequency of sister chromatid exchanges per C3H 10T½ chromosome plotted against drug concentration (0 to 6 mM) for chromosomes at the 2nd and 3rd mitotic divisions post incorporation of bromodeoxyuridine and misonidazole. The asterisk indicates that a 6 mM drug concentration did not allow any cells to progress into the 3rd division.

TABLE I
Chromosome Aberrations per Cell (Chr.Ab.) and Mitotic
Indices (MI) of Mouse C3H/10T_{1/2} Cells Following Exposure
to Misonidazole (standard errors in brackets)

Drug Contact Time (hr)	Concentration of Misonidazole (mM)							
	0		1		3		6	
	Chr.Ab.	MI(%)	Chr.Ab.	MI(%)	Chr.Ab.	MI(%)	Chr.Ab.	MI(%)
2	0.06	9.3	0.06	9.0	0.10 (0.02)	10.0	0.06	10.0
4	0.02	10.5	0.10 (0.04)	9.6	0.25 (0.04)	10.6	0.34 (0.08)	8.2
6	0.06	8.0	0.18 (0.05)	10.7	0.06 (0.01)	9.6	0.27 (0.07)	3.5
8	0.02	10.1	0.20 (0.03)	9.2	0.12 (0.06)	7.8	0.32 (0.12)	4.7
24	0.04	8.2	0.22 (0.07)	7.9	0.28 (0.09)	6.2	0.72 (0.26)*	0.5
48	0.05	7.4	0.19 (0.03)	6.5	0.23 (0.10)	6.2	---	<0.01
72	0.04	4.5	0.17 (0.05)	4.1	0.17 (0.05)	2.3	---	<0.01

*Only 30 scoreable metaphases were found in 3 experiments.

Fifty cells per time period were scored for 0 and 1 mM, and 100 cells were scored for 3 and 6 mM.

of cells in mitosis can indicate effects on cell cycle progression. It is evident that drug concentrations of 1 and 3 mM have effects that are comparatively negligible on the frequency of mitoses, while 6 mM misonidazole results in a substantial decline in the mitotic index with time. This finally results in no discernible mitoses after 48 or 72 hours of treatment, implying that cell progression through the cycle has ceased. Chromosomal aberrations show frequencies that are enhanced above control levels, though comparatively low, for all three drug concentrations. These data, contrasting the frequency of mitoses, the low frequency of chromosomal aberrations per cell and the substantial level of cell killing, imply that inter-phase cell death occurs, with a minor contribution from induced chromosomal aberrations and post-mitotic cell death. This was proposed previously as the basis of cell death following short term treatment of hypoxic cells.

Of the 247 aberrations found in the 1830 cells scored (~ 80 chromosomes per cell), only 39 involved chromatid interchanges (S or G2 phase, 2-break aberrations), while 8 were chromosomal interchanges (G1 phase, 2-break aberrations). The majority of the aberrations scored were simple chromatid deletions. The vast majority of aberrations seen represent events induced in the S and G2 phases of the cell cycle.

The manifestation of increased levels of sister chromatid exchanges (SCE's) in appropriately substituted chromosomes has been proposed as a sensitive endpoint to detect chromosome mutagenicity due to chemical agents. Certainly direct-acting carcinogens induce large increases in SCE frequency. Figure 2 illustrates the frequencies of SCE's found in C3H/10T $\frac{1}{2}$ chromosomes at the 2nd division and in previously unexchanged chromosomes at the 3rd division after treatment with 1, 3 and 6 mM misonidazole. It should be noted that 6 mM misonidazole did not allow any cells to progress into the 3rd division after initiation and comparatively few into the 2nd division. SCE levels in the 3rd division (1 cycle only) are approximately half that at the 2nd (2 cycles), as would be expected if conditions did not alter

during the course of the experiment. Also, the increase in exchanges with dose at the 2nd division is paralleled at the 3rd, indicating that this is a real increase and that the efficacy of the drug is not diminishing over the extended contact period. While the level of SCE's after treatment with 6 mM misonidazole is approximately two-fold greater than control, this level pales in comparison with the effect of commonly used chemotherapeutic agents where five to ten-fold increases are not uncommon.

Many chemotherapeutic agents that are known carcinogens *in vivo* are potent inducers of oncogenic transformation *in vitro*. The ability of a 1 mM concentration of misonidazole to induce transformation after contact time of 1, 3 and 6 days with C3H/10T_{1/2} cells is shown in Fig. 3. Miller and Hall have reported an extensive body of data on 3 and 6 day exposure of C3H/10T_{1/2} cells to misonidazole, and to misonidazole plus x-rays. A 1 mM concentration of misonidazole was chosen for intensive study since it may reflect that which is safely achievable in the clinic. Further, given the half-life of the drug in the body, a 1 day contact time may be a relevant estimate to the average pertaining to the clinical situation. The 0.0185% of transformants found after 1 mM drug for 1 day can be achieved in this cell system with a dose of 0.8 to 1 gray. Nevertheless, when contrasted with drugs which also induce low levels of transformation, such as vincristine, 6-mercaptopurine and methotrexate, these are either equivocal or not associated with carcinogenesis *in vivo*. Thus, on these grounds, the carcinogenic potential of misonidazole *in vivo* at clinically achievable concentrations is equivocal.

Chinese hamster V-79 cells

The toxic action of misonidazole against aerated and hypoxic cells maintained in ampules in the presence and absence of BUdR is shown in Fig. 4. The results of two experiments are combined and, clearly, the presence of BUdR in cells following two rounds of replication enhances the cytotoxic action of misonidazole. Whether

Figure 3: The frequency of oncogenic transformation per surviving C3H 10T½ cell (percentage on a logarithmic scale) after exposure of cells to 1 mM misonidazole for 1, 3 or 6 days. Standard errors are indicated.

Figure 4: The proportion of surviving Chinese hamster V-79 cells after treatment with misonidazole (4 hours) under both oxidic and hypoxic conditions in the presence and absence of bromodeoxyuridine (5 μM). Standard errors are indicated where possible.

this finding reflects a direct or a secondary effect on DNA in this instance is not known. However, the frequencies of chromosomal aberrations seen in cells surviving to mitosis are not compatible with DNA damage manifesting as chromosomal aberrations being the basis for the effect. Mitotic cells were accumulated over eight 2-hour periods after treatment. The mean SCE frequencies per chromosome, mean mitotic index and mean chromosomal aberrations per cell are shown in Fig. 5. Overall, there are dose-dependent increases in SCE's per chromosome and chromosomal aberrations per cell with the increases expressed more readily in hypoxic cells. Conversely, the mean mitotic index declines with drug concentration in hypoxic cells while it is basically unaffected in aerated cells. The level of chromosomal aberrations in cells collected over 16 hours after treatment (at least 1 cell cycle) is not compatible with the substantial difference in cell killing in aerated and hypoxic cells. These data for hypoxic Chinese hamster V-79 cells further affirm the contention that the majority of cells die in interphase and not as a result of induced chromosomal aberrations and post-mitotic cell death.

Conclusion

In both aerated and hypoxic Chinese hamster V-79 cells, as well as in aerated mouse C3H/10T $\frac{1}{2}$ cells, increasing misonidazole concentration results in greater frequencies of chromosomal aberrations per cell and in sister chromatid exchanges per chromosome.

Taken in conjunction with the effect of misonidazole in reducing the flow of cells through the cycle and the level of cell killing observed, it can be surmised that lethality mainly manifests in interphase with a minor contribution due to chromosomal aberrations and cell death from genetic inequality of progeny cells.

Both in aerated and hypoxic cells, the levels of SCE's while increased, are never more than twice the control level. It must be concluded that, using SCE's as an indicator of mutagenicity - carcinogenicity, misonidazole is inefficient

Figure 5: Mean sister chromatid exchanges (S.C.E.'s) per chromosome (upper panel), mean mitotic index (%) (center panel) and mean chromosome aberrations per cell (lower panel) versus misonidazole concentration (0. to 5 mM) for both oxic and hypoxic Chinese hamster V-79 cells. Standard errors are indicated. For the S.C.E.'s all standard errors were smaller than could be shown.

compared with common chemotherapy agents.

At clinically relevant concentrations (1 mM) and contact times (24 hours or less), misonidazole shows the following properties:

- a) Oncogenic transformation, *in vitro*, of 0.02% in mouse C3H/10T $\frac{1}{2}$ cells.
- b) Sister chromatid exchange frequencies less than 10% over control incidence in mouse C3H/10T $\frac{1}{2}$ cells and both aerated and hypoxic Chinese hamster V-79 cells.
- c) Over 90% of cells free of chromosomal aberrations in mouse C3H/10T $\frac{1}{2}$ cells and both aerated and hypoxic Chinese hamster V-79 cells.
- d) A similar rate of progression through the cell cycle as untreated cells in mouse C3H/10T $\frac{1}{2}$ cells and aerated Chinese hamster V-79 cells.
- e) Over 90% of cells clonogenically competent in mouse C3H/10T $\frac{1}{2}$ cells (even up to 72 hours drug contact) and in aerated and hypoxic Chinese hamster V-79 cells (4 hours drug contact).

While the carcinogenic and mutagenic potential of misonidazole is positively indicated, the frequency of both oncogenic transformations and of sister chromatid exchanges is small when compared with chemotherapeutic agents of known carcinogenic capacity.

RB-14 Antipain and radiation effects on oncogenic transformation and sister chromatid exchanges in syrian hamster embryo and mouse C3H 10T $\frac{1}{2}$ cells

C.R. Geard, M. Rutledge-Freeman, R.C. Miller and C. Borek

Introduction

A wide spectrum of proteases are manifested in concert with malignant transformation and tumorigenesis. This has led to the concept that these processes could perhaps be reduced if not prevented if proteolytic activity were hindered by the presence of added protease inhibitors. Protease inhibitors have been shown to inhibit tumorigenesis in mouse skin, to inhibit the growth of transformed cells, to block hormonal activation of enzymes and inhibit DNA synthesis and functions, as well as to inhibit bacterial mutagenesis and viral activation. A small peptide inhibitor derived from Actinomycetes, antipain (1-carboxy-2-phenylethyl) carbomoyl-L-arginyl-L-valylargininal) has been shown to inhibit error-prone (SOS) DNA repair of *Escherichia coli*, while AP also reduces radiation-induced-transformation.

However, this plethora of antioncogenic-mutagenic actions of protease inhibitors was confounded when it was found that the time of addition of AP dramatically altered the radiation-induced-transformation response of both mouse C3H/10T $\frac{1}{2}$ cl. 8 and Syrian hamster embryo cells. If AP was in contact with the cells at the time of irradiation, transformation frequencies were enhanced, whereas if it was added after irradiation, transformation was reduced, with later additions lessening the inhibitory effect. That is, there were two diametrically opposed modes of action and it was suggested that modification of gene action expressed as altered DNA repair may be involved.

This study was designed to address the question of whether the temporal modification of induced transformation response by antipain was paralleled by induced changes in sister chromatid exchange frequencies, a monitor of DNA interchange

activity. S.C.E.'s have been shown to be sensitive indicators of mutagen-carcinogens and to have considerable utility as monitors of such compounds.

Further, the earlier study involved the continuous treatment of cells with antipain from the time of addition to the time of scoring. Since a pre-irradiation treatment enhanced the response to radiation whilst a post-irradiation treatment reduced it, obviously the continued presence of AP could have given an incorrect impression of the enhancement. In this experiment AP was removed after irradiation and a further augmentation of transformation response was found, while the frequencies of SCE's did not correlate with the opposing actions of the AP in either cell system.

Method and Materials

Chemicals.

The water soluble protease inhibitor, antipain, was kindly donated by Dr. Walter Troll, and was added to the cell cultures in complete medium at a final concentration of 10 μ M (6 μ gm/ml).

BrdU (3 or 10 μ M)(Calbiochem-Berhing, La Jolla, MO) and colcemid (5 M) (Grand Island Biological Co., Grand Island, NY) were used in the SCE studies and care was taken to avoid exposing cells with BrdU to anything other than subdued natural or gold light.

Methods of irradiation.

Attached cells were irradiated at room temperature with a Siemens Stabilipan x-ray machine operating at 300 kVp and 12 mA, with 0.2 mm Cu filtration at a dose rate of 0.32 Gy min⁻¹.

Cell culture and transformation assay.

a) Mouse C3H/10T $\frac{1}{2}$ cl. 8 cells.

Cultures were maintained in Eagle's basal medium supplemented with 10% heat inactivated fetal bovine serum.

Cells from passages 9 to 14 were used throughout. Cells were plated into 100 mm dishes (Falcon) 18 to 24 hours before x-irradiation, with AP added at the time of plating or 10 mins. after irradiation and removed 24 hours later. Cell numbers were adjusted so that about 40 cells would produce clones for assessing plating efficiency and cell survival, and about 400 cells for the transformation assay. For assaying the frequency of transformations, cells were refed weekly for 5 weeks, when they were fixed and stained with Giemsa. The frequencies of darkly staining transformed foci overlying the confluent monolayer of normal cells were assessed by the criteria of Reznikoff et al. and only types 2 and 3 transformed foci recorded as transformants.

b) Primary Syrian hamster embryo cells.

Short term cultures of freshly explanted midterm hamster embryos were prepared and irradiated 24 hours after seeding 10^3 cells on 3×10^4 syngeneic feeder cells in 25 cm^2 flasks (Falcon). AP was added either at the time of seeding or 10 mins after irradiation and removed 24 hours later or left in contact with the cells for the two weeks of the experiment. After this time cultures were fixed, stained, and scored for transformation as described previously.

Transformed colonies were scored from among the surviving colonies, allowing an evaluation of plating efficiency, cell killing, and cell transformation in the same flasks.

Sister chromatid exchange assay.

Experiments assaying for SCE's were carried out at the same time as those for transformation. For both cell lines about 2×10^5 viable asynchronous log-phase cells per 100 mm dish were treated with BrdU ($3 \mu\text{M}$ for the 10T $\frac{1}{2}$ cells; $10 \mu\text{M}$ for the SHE cells) 24 to 26 hours before x-irradiation. Mitotic cells were colcemid accumulated from 6-12, 12-18, 18-24, and 24-30 hours after irradiation at which time they were fixed and air-dried slide preparations made.

Cells were stained in 2% Giemsa (Gurr's R-66) in 0.3 M Na_2HPO_4 at pH 10.4 (adjusted with 1 N NaOH) for 12 minutes to 2 hours. This staining procedure differentiates chromatids that have incorporated BrdU over two replication cycles (darkly stained) from chromatids that incorporated BrdU in one cycle (lightly stained). Cells that have been in BrdU for one, two or three replication cycles are readily assessed and the frequencies of each were recorded in mitotic cell samples of 200 from each treatment. The percentage of cells in mitosis (mitotic index) was also determined from samples of 1,000 cells from each treatment.

Sister chromatid exchanges were recorded on a per chromosome basis in samples of 2,000 chromosomes from each treatment from cells in their second metaphase after BrdU incorporation. That is, a comparison was made between cells which were maintained as controls or in AP for two replication cycles as well as cells that were irradiated in their second cycles and antipain added in that cycle. Radiation-induced SCE's are thus induced in one cycle only, whereas AP could have induced SCE's in both cell cycles, or in one cell cycle.

Results

Oncogenic transformation.

Table 1 presents the transformation data for both the SHE and 10T $\frac{1}{2}$ cells. AP alone has a negligible effect on cell survival in either cell system. While the radiation alone reduces survival, the addition of AP either before or after irradiation has no additional enhancing or deleterious effect.

However, the AP treatments show a consistent effect on the frequency of transformants. In both cell systems the presence of AP prior to and at the time of irradiation substantially enhances the transformation incidence, while its addition after irradiation and presence for 24 hours substantially reduces the frequency of transformation.

The continuous treatment conditions utilized previously were retested with the

TABLE 1

CELL TRANSFORMATION IN VITRO AFTER TREATMENT OF RODENT CELLS WITH X-RAYS AND ANTIPAIN

	Syrian hamster embryo cells					Mouse C3H 10T $\frac{1}{2}$ cl. 8 cells				
	Surviving fraction	Total colonies	No. of trans-formed colonies	Frequency of trans-formed colonies (x 10 ⁻³)	Ratio $\frac{\text{x-rays + AP}}{\text{x-rays alone}}$	Surviving fraction	Total cells	No. of trans-formed cells	Frequency of trans-formed cells (x 10 ⁻⁴)	Ratio $\frac{\text{x-rays + AP}}{\text{x-rays alone}}$
Control	1.00 (0.02) ^a	3,200	0	0	—	1.00(0.18) ^a 1.00(0.17)	7,200 4,800	0 0	0	—
Antipain (10 μ M)	0.92	4,010	0	0	—	1.00 0.91	14,400 6,100	0 0	0	—
X-irradiation ^b	0.59	4,130	24	5.81 $\pm$ 1.19 ^c	—	0.32 0.34	21,400 22,900	15 15	6.78 $\pm$ 1.24	—
Antipain and X-rays (AP added 24 hrs before and removed 10 mins after irradiation)	0.52	5,130	76	14.81 $\pm$ 1.70	2.55	0.30 0.43	18,100 21,700	25 31	14.05 $\pm$ 1.94	2.07
X-rays and antipain (AP added 10 mins after irradiation and removed 24 hrs later)	0.53	5,910	18	3.05 $\pm$ 0.72	0.52	0.30 0.39	18,600 31,200	5 9	2.79 $\pm$ 0.77	0.41

^aThe plating efficiency of control cells is given in brackets.^b3 gray of 300 kVp x-rays for Syrian hamster cells; 4 gray for mouse C3H 10T $\frac{1}{2}$ cells.^cStandard errors based on Poisson expectations.

SHE cells. AP was added to the cultures 24 hours before irradiation (3 Gy) and remained in contact with the cells for the two weeks of the experiment. With a surviving fraction of 0.49, there were 51 transformed colonies amongst the 5000 colonies scored. Hence, the ratio of the yield with x-rays plus AP over yield with x-rays alone was 1.76 which is in good agreement with the previously obtained value of 1.86. Therefore, removing the AP 10 min. after irradiation results in a 34% yield enhancement of transformation while a very similar 38% enhancement was obtained with the $10T\frac{1}{2}$ cells. The previous experiment utilized 3 Gy of x-irradiation of $10T\frac{1}{2}$ cells while the present experiments use 4 Gy. Although the absolute yields of transformants are increased the ratios are not affected so the opposed behavior of AP in both enhancing and reducing radiation induced transformation is near equally expressed at different radiation doses.

Sister chromatid exchanges.

Table 2 presents the mean frequencies of SCE's per chromosome from 5 replicate experiments with the $10T\frac{1}{2}$ cells and 4 replicate experiments with the SHE cells. While there was variability between the experiments, overall, for both cell systems AP slightly enhances SCE frequencies, while the 3 Gy dose of x-rays results in approximately a doubling of SCE's. The addition of AP to the irradiated cells after irradiation results in no consistent change in SCE's, however, the presence of AP at the time of irradiation reduces SCE frequencies in the hamster chromosomes, while the mouse SCE's are unaffected. Certainly though, neither cell system shows an additive effect of AP on irradiation induced SCE's. When AP was added to untreated cells after the time of irradiation such that it was in contact with cells in the one cell cycle before fixation (as opposed to the two cell cycles when it was added initially) there was also a slight though inconsistent enhancement of exchanges.

The relative frequencies of first, second or third division mitoses found after the sequential accumulations and fixations was unchanged in the presence of AP.

TABLE 2

MEAN FREQUENCIES OF SCE'S PER CHROMOSOME IN RODENT CELLS AFTER TREATMENT WITH X-RAYS AND ANTIPAIN

	<u>Syrian hamster embryo cells^a</u>				<u>Mouse C3H 10T$\frac{1}{2}$ cl. 8 cells^a</u>				
Control	0.29 ^b	0.38	0.34	0.30	0.29	0.20	0.22	0.23	0.26
Antipain ^c (10 μ M)	0.39	0.44	0.37	0.35	0.31	0.21	0.31	0.33	0.34
X-rays (3 Gy)	0.77	0.88	0.69	0.56	0.38	0.46	0.48	0.51	0.44
Antipain and X-rays (AP added 24 hrs before and removed 10 mins after irradiation)	0.64	0.67	0.53	0.48	0.42	0.45	0.51	0.50	0.47
X-rays and ^c antipain (AP added 10 mins after irradiation)	0.88	0.98	0.58	0.58	0.42	0.34	0.56	0.41	0.44
Antipain ^c (added to control cells 10 mins after the time of irradiation)	0.46	0.41	0.35	— ^d	0.22	0.21	0.33	0.31	0.29

^aMean frequencies are given from 2,000 chromosomes per treatment in 4 replicate experiments with SHE cells, and 5 replicate experiments with 10T $\frac{1}{2}$ cells.

^bStandard errors of the mean were less than 8% of the mean in all cases.

^cAntipain was left in contact with cells until fixation; up to 30 hrs.

^dMishandled

After irradiation (in both cell systems) AP treatments had no further influence on mitotic delay, and the yield of mitoses or the frequencies of the different divisional stages at the different fixations was close to control frequencies or to those found after radiation alone.

It should be noted, however, that the SHE cells were much more variable in their cycling behavior than the 10T $\frac{1}{2}$ cells. Even at the later fixations (48 and 54 hours after initiation) when substantial frequencies (up to 78%) of third division mitoses were found, there were still about 5% of first division mitoses. Also there were some third division mitoses ($\sim$ 5%) at 36 hours after culture initiation. That is, while many hamster cells progressed through 3 cell cycles in 36 to 54 hours, a small fraction only moved through one cycle. Given the heterogeneous origin of these cells and their comparatively short time in culture such variability is not surprising. This variability was not found with the 10T $\frac{1}{2}$ cells, where in control (or antipain treated) cells there were virtually no second division cells 24 hours after the time of irradiation. There were still about 15% of second division cells (with the remainder 3rd divisions) in cells that were irradiated, 30 hours after irradiation, indicative of the radiation-induced mitotic delay. Overall, at the concentration used (10 μ M) AP was not cytotoxic, nor did it effect cell growth rate or progression through the cell cycle.

The dramatic contrast between the effect of AP on radiation-induced oncogenic transformation and SCE's is shown in Chart 1. The histogram panels on the left show the overall mean SCE frequencies found in both cell systems, while the histogram panels on the right show the mean transformation frequencies. There were no transformants in either the control or AP treated cells, whereas both control and AP treated material express SCE's. The substantial variation in yield of transformants with time of AP application is not paralleled by changes in SCE frequencies.

Chart 1

A histogram contrasting the effects of x-rays and antipain on SCE's per chromosome and oncogenic transformations. The mean frequencies $\pm 2 \times \text{S.E.}$ are shown for SCE's in the left panels. The right panels show the mean frequencies $\pm \text{S.E.}$ of oncogenic transformation. Antipain was added 24 hr. before and removed 10 min. after x-irradiation (antipain + x-ray) or added 10 min. after x-irradiation and removed 24 hr. later (x-ray + antipain). 3 Gy. of x-rays was delivered for SCE studies and for transformation of SHE cells, with 4 Gy. for transformation of 10T $\frac{1}{2}$ cells.

Discussion

These observations confirm and expand on previous findings from this laboratory in showing that the action of AP in enhancing radiation-induced transformation is augmented if AP is removed after irradiation. The yield enhancement was 34% and 38% respectively for the SHE and 10T $\frac{1}{2}$ cells over that obtained previously when AP was in contact with the cells throughout. Further, with regard to the post-irradiation reduction of transformation, a 24 hour treatment with AP reduces transformation to 0.5 and 0.4 that of radiation alone, similar to the earlier study in which there was continuous cell contact with AP following addition 10 min. after irradiation. Therefore, a 24 hour post irradiation treatment is as effective in reducing transformation as a long term treatment, while a 24 hour pre- and during irradiation treatment is more effective at enhancing transformation than a long term treatment.

The finding that a 24 hr post irradiation treatment is as effective in reducing transformation as a continuous treatment mitigates against the proposal that protease inhibitors exert their effects by interacting with the induced proteases of transformed cells or of selectively influencing the growth of transformed cells. A 1 day treatment with AP does not even encompass the time necessary for the fixation of the damage induced by the radiation. Further, it seems highly unlikely that protease-allowed mobility or modification of contact inhibition can play a role when the effects of the AP are so closely associated with the induction rather than the expression phase of transformation.

While it is not unreasonable to ascribe the contradictory actions of AP to modifications of DNA repair processes, such actions are certainly not reflected in modifications of the incidence of SCE's. Varying the time of addition of AP either had no influence on SCE frequencies or for the SHE cells resulted in a reduction in SCE frequencies when AP was present at the time of irradiation.

Overall, while differential effects of AP in influencing DNA repair processes in these cells cannot be ruled out, these data show that this effect does not concomitantly influence SCE frequencies, which are sensitive indicators of DNA damage. In agreement with our earlier observations on the effects of retinoids and TPA; while SCE's are highly useful as indicators of primary carcinogens, they do not appear to reflect subsequent modification by secondary agents.

The utility of AP as an agent for the reduction of tumorigenesis is brought into question, since quite short temporal differences in application can result in near five-fold differences in the frequencies of oncogenic transformations, ranging from substantial enhancement to substantial reduction.

RB-15 Interaction of high- and low-LET sublethal damage: effect on the reproductive capacity of cultured human cells.

P.S. Furcinitti

Introduction

Much interest has been expressed recently concerning the interaction of radiations of different qualities (1-3). Some work suggests that a clinically useful gain may result from using a mixture of radiations compared with x-rays alone; for example an initial dose of pions or neutrons followed by x or gamma rays (4). The existence of sublethal damage following irradiation with high LET radiation, and its interaction with sublesions produced by low-LET radiation has been demonstrated for cultured mammalian cells of rodent origin (5-8). However, pioneering work by Barendsen et al. (9) in which cultured human (kidney cells T-1) were irradiated by alpha particles followed by x rays indicates independent cell killing by sequential exposures to high and low radiations. The unique track segment and x-ray irradiation facilities available at RARAF provide an ideal opportunity to investigate this problem since doses of high- and low-LET radiations may be delivered to cells with short time intervals between doses. In initial experiments to investigate the discrepancy between the response of rodent and human cells *in vitro* to mixed modality irradiation, cultured human kidney T-1 cells were exposed to ^3He ions of well-defined LET (70, 90, or 140 keV/micron) followed five minutes later by graded doses of 50 kV x rays.

Materials and Methods

Human kidney T-1E cells were maintained by continuous subculture in flasks with Eagle's minimum essential medium (MEM) plus 10 percent fetal bovine serum and 100 $\mu\text{m}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. This mixture will be called

EM-10. Cells were incubated in a 5 percent CO₂ incubator at a relative humidity of approximately 95 percent at 37°C. Asynchronous cells were plated on special 6 µm mylar dishes 1 to 2 days prior to irradiation. Immediately after irradiation cells were trypsinized, counted, and appropriate dilutions made for replating in 100 mm tissue culture dishes containing 10 ml of EM-10 and left undisturbed for 10-14 days for colony formation. Irradiation facilities and procedures have been previously described (10). Complete survival curves for high-LET, low-LET, and mixed LET irradiations were obtained in each experiment.

Results and Analysis

Figure RB-15-1 shows cell survival data for graded doses of x-rays, for graded doses of ³He ions having an LET of 70 keV/µm, and for single doses of helium ions followed by graded doses of x-rays. Figures RB-15-2 and RB-15-3 show the results of similar experiments with helium ions of 90 and 140 keV/µ, respectively. It is clear qualitatively that as the LET increases, there is a tendency towards independent action of the two types of radiation.

In order to assess quantitatively the amount of interaction between high- and low-LET doses on survival, an interaction factor (IF) may be defined as:

$$IF = \frac{D_{IND} - D_{INT}}{D_{IND} - D_{High\ LET}}$$

where D_{IND} = total of both high- and low-LET radiation dose to achieve a given survival level if the high- and low-LET radiation act independently; D_{INT} = total dose actually given to achieve the same survival level as produced

by D_{IND} ;

$D_{High\ LET}$ = dose of high-LET alone actually given to achieve this survival level.

This factor is normalized such that if the mixed modality survival curve is

Figure 1: Fraction of asynchronous human kidney T-1 cells surviving graded doses of x rays, ^3He ions (70 keV/ μm) and 2.10 Gray of ^3He ions followed by graded doses delivered 5 minutes later. The curve labeled independent survival is the x-ray survival curve with its origin shifted to coincide with the survival level produced by the initial dose of ^3He ions.

Figure 2: Cell survival for T-1 cells exposed to ^3He ions (90 keV/ μm), x rays and 1.5 Gray of ^3He ion plus x rays. The curve labeled independent survival was drawn as indicated in the figure caption of 1.

Figure 3: Fraction of T-1 cells surviving exposure to ^3He ions (140 keV/ μm) x rays, and 1.5 Gray of ^3He ions followed by x rays. The independent survival curve is explained in figure caption 1.

coincident with the high-LET curve the value of IF = 1.0 and if the mixed modality curve is coincident with the curve for independent survival IF = 0.0. Figure RB-15-4 shows a plot of this ratio as a function of the LET of track segment particles. A survival level of 0.02 was chosen and the doses needed to calculate IF were determined for each LET value.

Discussion

The data in Figure RB-15-4, although limited, may be fitted approximately by a straight line. Extrapolation to higher LET values suggests that by about 172 keV/ μ m, mixed modalities may act independently. Extrapolation to low values suggests that the interaction produced by ^3He ions having an LET of ~ 18 keV/ μ m and x rays is such that the survival curves for the mixed radiation and high-LET curves might be coincident. Further investigations should be undertaken at LET values other than those used in this study and synchronous cells employed to explore cell cycle dependence for these mixed field irradiations.

References

1. Railton, R., Lawson, R.C., and Porter, D. (1975). Interaction of gamma ray and neutron effects on the proliferative capacity of Chinese hamster cells. *Intern. J. Radiat. Biol.* 27, 75-82.
2. Duran, R.E. and Olive, P.L. (1976). Irradiation of multi-cell spheroids with fast neutrons versus x-rays: A qualitative difference in sublethal damage capacity or kinetics. *Int. J. Radiat. Biol.* 30, 589-592.
3. Ngo, F.Q.H., Han, A., and Elkind, M.M. (1977). On the repair of sublethal damage in V79 Chinese hamster cells resulting from irradiation with fast neutrons or fast neutrons combined with x rays. *Int. J. Radiat. Biol.* 32, 507-511.
4. Yuhas, J.M., Li, A.P., and Kligerman, M.M. (1979). Present status of the proposed use of negative Pi mesons in radiotherapy. In Advances in Radiation Biology, Vol. 8, 51-83.
5. Ngo, F.Q.H., Blakely, E.A., and Tobias, C.A. (1978). Does an exponential survival curve of irradiated mammalian cells imply no repair? 26th Annual Meeting of the Radiation Research Society, Toronto.

Figure 4: Interaction factor vs. LET of ^3He ions. Doses used to calculate this factor were those necessary to produce a survival of 0.02. The curve is a least-squares fit to a straight line.

6. Ngo, F.Q.H., Blakely, E.A., and Tobias, C.A., and Chang, P.Y. (1979). High LET radiations produce sublethal lesions. 6th International Congress of Radiation Research, Tokyo.
7. Li, G.C., Hahn, G.M., Fisher, G.A., Fessenden, P. and Bagshaw, M.A. (1979). Interaction of negative Pi-mesons with x-irradiation. 6th International Congress of Radiation Research, Tokyo.
8. Bird, R.P., Rossi, H.H., Rohrig, N., Marino, S.A., Zaider, M., and Mills, R.E. (1980). Cell inactivation by a mixture of high- and low-LET radiations. Abstract, 28th Annual Meeting of the Radiation Research Society, New Orleans.
9. Barendsen, G.W., Beuskher, T.L.J., Vergroesen, A.J., and Budke, L. (1960). Effects of different ionizing radiations on human cells in tissue culture. II. Biological experiments. Radiat. Res. 13, 841-849.
10. Bird, R.P., Rohrig, N., Colvett, R.D., Geard, C.R., and Marino, S.A. (1980). Inactivation of synchronized Chinese hamster V79 cells with charged-particle track segments. Radiat. Res. 82, 277-289.

P. Goldhagen, M. Freeman, and E.J. Hall

^{125}I Iodine sources have been increasingly used for permanent implants in the treatment of some types of human cancers. ^{125}I has a half-life of 60.2 days and emits photons with energies between 27 and 35.4 keV. The initial minimum dose rate in a typical implant is about .08 gray/hour. The dose rate is higher near each source and lower as the isotope decays. Tumor responses observed clinically have been greater than was expected at such low dose rates from experience with higher-energy photon emitting radionuclides (1). Although some measurements of the RBE of soft photons have been made (2-4), none has been made on mammalian cells using measured, clinical-range dose rates. The importance of dose rate for RBE has been discussed by Hall (5).

This laboratory is beginning low dose-rate studies of the radiobiology of ^{125}I photons by comparing their effectiveness in producing reproductive death and mitotic delay in Chinese hamster ovary (CHO) cells *in vitro* with that of ^{137}Cs gamma rays. The procedures and analysis will be similar to those previously used in this laboratory to measure the biological effectiveness of neutrons from ^{252}Cf relative to gamma rays from radium (6). Work during the past year has concentrated on dosimetry and on development of facilities to simultaneously provide conditions suitable for cell growth and clinical-range dose rates from ^{125}I or ^{137}Cs . The dosimetry work is described in section PD- 2 of this Progress Report (7).

To avoid large uncertainties in the dose absorbed by cells growing on polystyrene when they are exposed to the low energy photons from ^{125}I (7), we

are not using commercial cell culture dishes. Instead, we have made our own 35 mm petri dishes from polyvinylidene fluoride (PVDF), a plastic which is nearly tissue equivalent for ^{125}I photons. CHO cells are grown in these dishes in 2.1 ml of McCoy's 5A medium containing 20% heat-inactivated calf serum, 100 units/ml of penicillin G sodium and 100 mcg/ml of streptomycin sulfate. CHO cells are used because their radiobiological properties are well known and because they grow well without a specially-treated attachment surface. Figure RB-16-1 shows a typical growth curve for these cells in our PVDF dishes. There is an initial lag period, a period of exponential growth with a population doubling time of approximately 14 hours, and a plateau. The same growth pattern and doubling time are observed when the cells are grown in Falcon T-25 flasks.

^{137}Cs exposures are carried out by means of a single planar array of ^{137}Cs sources, fixed in a plastic holder which is supported horizontally in the center of a water-jacketed plastic cell-irradiation box. The box is surrounded by thermal insulation and lead shielding. A push rod attached to the back of the source holder allows it to be removed through a slot in the back of the box into a separately shielded compartment, permitting safe access to the cell culture dishes. Four of the PVDF dishes at a time can be supported on each of up to 12 shelves. The shelves are spaced in pairs, above and below, at distances from 2 to 10.5 cm from the source plane, allowing 8 dishes to be irradiated at each of up to 6 dose rates. By removing some of the dishes at different times a range of doses, and hence a survival curve, can be obtained for each dose rate.

The ^{137}Cs irradiation facility is shown in Fig. RB-16-2. The front door of the lead shielding and most of the thermal insulation were not yet installed when the photograph was taken.

Figure 1: Growth curve for CHO cells in our polyvinylidene fluoride petri dishes.

Figure 2: ^{137}Cs irradiation facility.

^{125}I exposures will be carried out using 3 separate 34 mm circular arrays of "seed" sources. Each array is used to irradiate a stack of 5 PVDF 35 mm petri dishes; 3 above the source plane and 2 below. This arrangement provides 6 dishes at a low dose rate, 6 at a medium rate, and 3 at a high rate. Each array of seeds is supported between 2 layers of Mylar adhesive tape stretched across a plastic holder. Similar plastic holders, without tape, are used to support the petri dishes at their edges. The sources and dish stacks are housed in the lower 3 sections of a water-jacketed brass box inside which cell incubation conditions are maintained. Fig. RB-16-3 shows the box before it was surrounded with thermal insulation. Three upper sections are shielded from the sources and house a total of 18 control dishes. The source holders can each be slid into separate shielded compartments at the rear of the box so that the dishes may be safely handled.

We have not placed the irradiation fixtures for both isotopes in a single commercial incubator because the lead shielding for the ^{137}Cs would have crushed available incubators. Instead, extensive precautions have been taken to keep the two irradiation boxes at identical conditions of temperature, gas composition, and humidity. A single constant-temperature water circulator supplies both boxes with 37.4°C water, and a single source of humidified air- CO_2 mixture supplies both boxes with gas. The ^{125}I box is located on top of the shielding for the ^{137}Cs box. Initially there were some variations of the temperature in the ^{137}Cs box, but these have been eliminated by using thermistor-controlled heaters to maintain the lead shielding at 37.4°C . Growth curves for CHO cells in both boxes (with the sources withdrawn) and in commercial incubators are indistinguishable.

Fig. RB-16-4 shows growth curves for CHO cells in PVDF dishes irradiated

Figure 3: ^{125}I irradiation facility.

Figure 4: Growth curves for CHO cells irradiated at 3 different dose rates in the ^{137}Cs facility and not irradiated in the control section of the ^{125}I box.

at 3 different dose rates in the ^{137}Cs facility and not irradiated in the control section of the ^{125}I box. The dose rates shown, .78, .27, and .12 gray/hour, are computer-calculated values (7). The doubling time of the exponentially growing unirradiated cells in this experiment was 12 hours. All dishes were incubated for 40 hours after inoculation to allow the cells to reach exponential growth before irradiation began. At .12 gray/hour, the rate of cell reproduction was greater than the rate of cell death, while at .27 gray/hour the reverse was true.

Figure RB-16 -5 shows curves of the number of viable cells as a fraction of control vs. dose. The data were taken from the previous figure. These survival curves are basically exponential as expected.

Preliminary work necessary to establish the soundness of our cell culture techniques and facilities is now complete. We will begin cell irradiations for the RBE measurement as soon as we have finished measurements of the dose rates from the first batch of ^{125}I sources.

REFERENCES

1. Kim, J.H., Hilaris, B.S. Clinical and radiobiological consideration of iodine-125 implants. Afterloading: 20 years of experience, 1955-1975, Proc. 2nd Int. Symp. on Radiation Therapy: 59-61 (1975).
2. Zeitz, L., Kim, S.H., Kim, J.H., Detko, J.F. Determination of relative biological effectiveness (RBE) of soft x-rays. Radiat. Res. 70: 552-563 (1977).
3. Hering, E.R., LeRoux, P.L.M., Sealy, R. A comparison of the biological effect of iodine 125 and tantalum 182 gamma rays using *Vicia Faba* roots. Br. J. Radiol. 51:392-393 (1978).

Figure 5: Plot of the number of visible cells as a fraction of control vs. dose from ^{137}Cs at 3 different dose rates. The data were taken from Fig. 4.

4. Hering, E.R. A comparison of the biological effect of ^{125}I and ^{192}Ir gamma rays on the roots of *Vicia faba* using a specially designed applicator. Br. J. Radiol. 53:255-258 (1980).
5. Hall, E.J. Radiation dose-rate: a factor of importance in radiobiology and radiotherapy. Br. J. Radiol. 45:81-97 (1972).
6. Hall, E.J., Rossi, H.H., Roizin, L.A. Low dose-rate irradiation of mammalian cells with radium and californium 252. Radiology 99:445-451 (1971).
7. Goldhagen, P. Dosimetry and source design for measurement of the relative biological effectiveness of photons from ^{125}I , USDOE Progress Report C00-4733-3 section PD- 2 (1980).

RB-17 Sequential exposures with high- and low-LET radiations

R.P. Bird

Experiments were continued using the RARAF track segment facility in which synchronized cells were irradiated with high-LET charged particles and then, within a few minutes, were exposed to graded doses of x rays. The intent of these experiments is to evaluate the interaction of damage produced by radiations of different quality. The techniques for irradiation and for obtaining Chinese hamster V79 cell synchrony by hydroxyurea treatment were described previously (1,2). Radiations with three LET values were used for the charged particle irradiations: 50 keV/ μ m deuterons and helium-3 ions of 90 and 160 keV/ μ m. The 50 kV x-ray generator, mounted alongside the track segment beam line, was the source of x rays used primarily in the sequential irradiations. The time interval between the charged-particle irradiation of a dish of cells and a subsequent x-ray exposure was five minutes except for a few cases where the interval was up to seven minutes.

The results of these experiments are shown in the composite Figs. RB-17-1 and -2 for the G₁/S transition cells and the late-S phase cells, respectively. The shaded areas in each case are bounded by upper curves that would result from independent radiation effects. That is, the surviving fraction for independent effects would be simply the product of the fractions resulting from the high-LET dose and from the low-LET dose. The lower bounds are curves drawn approximately through the actual data. Therefore, the shading indicates the extent of lethal interaction between the two radiations. It is clear in this series of experiments that there is damage interaction with subsequent x-ray exposures for each LET value and dose examined.

Zaider and Rossi (3) have provided a theoretical basis for this damage interaction. The additional cell killing is obtained by an interaction term,

$$\exp[-2\sqrt{\beta_L}\sqrt{\beta_X}D_LD_X] \quad (1)$$

Figure 1: Survival curves for synchronized G₁/S transition cells irradiated with x-rays only, or high-LET charged particles only, or with graded doses of x-rays (closed symbols) following within a few minutes a dose of high-LET radiation. The charged particles and their average LET values are indicated in each panel. The same symbol in each panel denotes results from one experiment with one standard deviation indicated.

in which the doses and damage for each radiation are subscripted with L or X for high-LET or x ray. In Fig. RB-17-2, for late S phase cells, values of β_X and β_L were determined from the survival curves obtained for each radiation separately and were used with Eq. (1) to evaluate the interaction. This evaluated interaction was used to determine the lower bound to the shaded areas in the figure, showing general agreement with the experimental results. In Fig. RB-17-1 for G_1/S cells, the lower bounds of the shaded areas were drawn according to Eq. (1) using the measured value of β_X and assumed values for β_L . The estimation of β_L values obtained by using Eq. (1) directly, resulted in data which is plotted in Fig. RB-17-3 as stars. The other data points are reproduced from the previous Annual Report (Ref. 4, Fig. RB-7-2) and obtained from survival curve data by computer analysis. The new data extend the LET trend of the survival curve parameters to the region of high LET where evaluation directly from the survival curves resulted in values that could not be distinguished from zero.

A qualitative description of the interaction damage between two radiations is shown in Fig. RB-17-4. When $\beta_L = \beta_X$, the shape of the subsequent x-ray survival curve is the same as that for x-irradiation alone for x-ray doses beginning with the same dose as that of the high-LET radiation. The comparison of shapes is made by shifting the subsequent x-ray curve vertically to the surviving fraction for $D_X = D_L$. If the high-LET damage for subsequent x-ray interaction is substantially greater or less than the same dose of x rays would provide, then such a comparison of shapes will show it. In Fig. RB-17-5, the curve-shape comparison indicates approximate equality of damage per dose of two radiations (helium-3 ions and 50 kV x rays). Such a result is similarly obtained for the other experimental results with the exception of the comparison shown in Fig. RB-17-6 for G_1/S cells exposed to deuterons (50 keV/ μ m) and x rays. In this case, it is apparent that $\beta_L > \beta_X$.

Figure 2: Survival curves for synchronized late-S phase cells obtained similarly to those described for Fig. 1.

Figure 3: The LET trends of survival curve parameters for cells synchronized at the G₁/S transition (left panel) and late-S phase (right panel). The stars indicate the product of LET and the square root of the estimated quadratic dose coefficients, for G₁/S cells, based on the interaction effect of high- and low-LET radiations described by Eq.(1).

Figure 4: A schematic representation of interaction between high-LET and low-LET radiations. The open circles represent renormalization of the "mixed radiation" data (closed circles) to the surviving fraction for a dose of x-rays the same as the high-LET dose. If $\beta_L = \beta_x$, the renormalized data fall on the "x-ray only" curve. If $\beta_L < \beta_x$ or $\beta_L > \beta_x$, the renormalization data fall to the right or to the left of the "x-ray only" curve, as indicated.

Figure 5: Renormalized survival data (closed symbols) for G₁/S cells irradiated with a dose of helium ions followed by graded x-ray doses. The circles denote an experiment using 90 keV/μm helium ions and/or x-rays. The squares denote an experiment using 160 keV/μm helium ions and/or x-rays.

Figure 6: Renormalized survival data (closed symbols) for G₁/S cells irradiated with a dose of deuteron ions followed by graded x-rays doses. The different symbols denote separate experiments.

Clinical trials of "mixed modality" radiation therapy are being reported. Doses to be used in such treatment schedules must accommodate the interaction effect when the combination is given in sufficiently short intervals. A good approximation of the interaction can be simply obtained by assuming subsequent x-ray lethality according to the dose delivered as high-LET radiation, as depicted in Fig. RB-17-5.

References

1. Bird, R.P. (1978). Track segment experiments: cell survival data. Progress Report COO-4733-1 (H.H. Rossi and E.J. Hall, eds.).
2. Bird, R.P., Rohrig, N., Colvett, R.D., Geard, C.G., and Marino, S.A. (1980). Inactivation of synchronized Chinese hamster V79 cells with charged-particle track segments. Radiat. Res. 82, 277-289.
3. Zaider, M. and Rossi, H.H. (1980). Additivity of the effects of different radiation. In press, Radiat. Res.; Sequential exposures to mixed fields of radiation--a theoretical approach. To be presented at the 7th Symposium on Microdosimetry, Oxford, England, September 8-12, 1980.
4. Bird, R.P. and Kliagua, P.J. (1979). Irradiations with the track segment facility and the analysis of data. Progress Report COO-4733-2.

The experiments with correlated ions were continued at RARAF using synchronized Chinese hamster V79 cells. Previous experiments measured the cell killing effectiveness of single- and multiple-ions using diatomic deuterium (1,2) or diatomic hydrogen (4) as the source of associated ions. Triatomic hydrogen was used in the present experiments. Table RB-18-1 lists the physical parameters of the irradiation. It will be noted that, with the thickest Mylar, the LET of the individual ions did not match that for the lesser Mylar thicknesses. This was a consequence of the limitation of the Van de Graaff accelerator.

The techniques of culturing and synchronizing the cells, and of irradiating and replating them for colony formation have been described previously (2,3) and remain unchanged. The measure of the effectiveness of correlated ions in these studies is the comparison of the surviving fractions obtained when irradiating cells with the same dose delivered either as single (uncorrelated) ions or as triple (correlated) ions. The surviving fractions are measured in one experimental setup for direct comparison as the survival ratio.

The results of survival ratio measurements for late-S phase cells, are shown in Fig. RB-18-1 for three different Mylar thicknesses which were used to achieve differences in the pairwise separations of ions as they traversed cells. Based on the curves drawn through the data (fitted by eye) a calculation of the ratio of linear coefficients was made according to the relationship (2)

$$\frac{\alpha_{3P}}{\alpha_P} = 1 + \frac{\ln(SR)}{\alpha_P D}, \quad (1)$$

where (SR) is the survival ratio for a dose, D. The linear coefficients for the single ion irradiations and for helium-3 irradiations were evaluated from survival

Table RB-18-1

Physical parameters for associated ions*

Accelerated ion/energy (MeV)	Mylar thickness	LET (keV/ μ m) at 3 μ m cell depth	Pairwise characteristic separation (nm)		
			<u>Mylar surface</u>	<u>3 μm cell depth</u>	<u>6 μm cell depth</u>
P ₃ /3.51	6 μ m	29	140	250	390
P ₃ /3.84	10 μ m	29	260	400	550
P ₃ /4.31	19 μ m	32	630	810	1040
³ He/4.9	6 μ m	82	-	-	-

*data obtained from Dr. N. Rohrig, BNL

Figure 1: Survival ratios for synchronized late-S phase cells comparing the effectiveness of single and triplet protons delivered at the same dose. The curves drawn through the data, with one standard error indicated, were fitted by eye.

curves shown in Fig. RB-18-2. Fig. RB-18-3 shows the ratio of linear coefficients, for triple protons vs. single protons as a function of characteristic separation and for helium ions vs. single protons where helium ions were used to approximate zero separation of triple protons. In the case of helium-ion irradiation, the LET achieved was less than the design value, about 82 keV/ μ m instead of 87 keV/ μ m (thrice the single ion LET value). Also indicated are the previously obtained results with diatomic deuterons (2).

The measurements using triple protons have demonstrated the effect of associated ions at substantially increased separations.

References

1. Bird, R.P. (1977). Molecular ion experiment: cell survival results. Annual Report on Research Project COO-3243-6 (H.H. Rossi and E.J. Hall, eds.).
2. Bird, R.P. (1979). Biophysical studies with spatially correlated ions.
3. Cell survival studies using diatomic deuterium. Radiat. Res. 78, 210-233.
3. Colvett, R.D. and Rohrig, N. (1979). Biophysical studies with spatially correlated ions. 2. Multiple scattering, experimental facility, and dosimetry. Radiat. Res. 78, 192-209.
4. Bird, R.P. (1979). The associated ion experiment. Progress Report COO-4733-2 (H.H. Rossi and E.J. Hall, eds.).

Figure 2: Survival curves for synchronized late-S phase cells irradiated either with single protons or with helium-3 ions.

Figure 3: The ratio of linear dose coefficients for synchronized late-S cells as a function of the characteristic pairwise separation of associated triple protons. Also shown (closed circles) are previously reported results obtained with associated pairs of deuterons.

TEACHING

Each month of the academic year, three members of the staff contributed a series of radiobiology lectures to medical students taking the general Radiology elective. Lectures were also given to second year medical students in the "Human Oncology" course and to third year students in the "Introduction to the Patient" course. In addition, a laboratory elective in "Cellular and Radiation Biology" was offered for medical students.

During the Fall, a course in Radiobiology was given for residents in both therapy and radiodiagnosis. Residents from Columbia-Presbyterian, affiliated hospitals and other institutions in the New York area attended.

Two graduate students are at present pursuing research for their dissertations in this laboratory under the supervision of Dr. Eric Hall.

Members of the staff participated in graduate teaching in the Departments of Pathology and Physiology of Columbia University and also as guest lecturers in other institutions in the city.

INVITED SEMINARS AND PAPERS AT SCIENTIFIC MEETINGS

Dr. Richard P. Bird

Annual Meeting of the American Society of Therapeutic Radiologists,
New Orleans, LA, October 1979

Annual Meeting of the Radiation Research Society,
New Orleans, LA, June 1-5, 1980

Dr. Carmia Borek

University of Albuquerque, New Mexico, Department of Radiology
July 1979

Los Alamos National Laboratory, July 1979

Conference on Cancer Biology, Institute of Pathobiology, Aspen,
Colorado, July 1979.

Weizmann Institute, Department Genetics, August 1979

New York Academy of Sciences Conference on "Differentiation and
Carcinogenesis in liver cell cultures", October 1979

American Society Cell Biology, Toronto, Canada, November 1979

New York Academy of Sciences Conference on Modulation of Cellular
Interactions of Vitamin A and derivatives (retinoids), March 1980

American Cancer Society Science Writers Conference, Daytona Beach, March 1980

Symposium "Carcinogenesis: fundamental mechanisms and environmental effects"
Jerusalem, Israel, April-May 1980

University of Vermont, Department of Pathology, May 1980

"Visiting Professor" University of Rochester, Department of Radiology &
Biophysics, May 1980

Annual Meeting American Society Cancer Research, San Diego, May 1980

Annual Meeting Radiation Research Society, New Orleans, June 1980

Dr. Michael L. Freeman

Thomas Jefferson University, Department of Radiation Therapy and Nuclear
Medicine, Philadelphia, PA, April 28-29, 1980

Third International Conference on Hyperthermia, Colorado State University
Fort Collins, CO, June 22-24, 1980

Dr. Paul Furcinitti

Annual Meeting Radiation Research Society, New Orleans, LA, June 1-5, 1980

Dr. Charles R. Geard

CROS Conference on Combined Modality of Cancer Treatment: Radiation
Sensitizers and Protectors, Key Biscayne, Florida, October 3-6, 1979

Annual Meeting Radiation Research Society, New Orleans, LA, June 1-5, 1980

Symposium in Radiological Technology, Columbia-Presbyterian Medical Center, NY
June 26, 1980

Dr. Paul Goldhagen

Annual Meeting Radiation Research Society, New Orleans, LA, June 1-5, 1980

Mr. Leon Goodman

Annual Meeting Radiation Research Society, New Orleans, LA, June 1-5, 1980

Dr. Eric J. Hall

American Board of Radiology Meeting, Key Biscayne, Florida, September 19-21, 1980

Electron Beam Symposia, Memorial Hospital, NY, NY, September 25-26, 1980

CROS Radiosensitizer Meeting, Key Biscayne, Florida, October 3-6, 1980

Annual Meeting of the American Society of Therapeutic Radiologists, New
Orleans, LA, October 23-26, 1980

Meeting to plan Federal Strategy for Radiation Research, Washington, D.C.,
November 6-7, 1980

Seminar, Brown University, Rhode Island, November 14-15, 1980

Annual Meeting of the Radiological Society of North America, Atlanta,
Georgia, November 26-28, 1980

Meeting of the Pacific Endocurietherapy Society, Mazatlan, Mexico,
December 4-7, 1980

RTOG Semi-Annual Meeting, Albuquerque, New Mexico, January 22-25, 1980

Seminar, New York Cancer Society, NY, NY February 6, 1980

Seminar, Colorado State University, Fort Collins, Colorado, February 11, 1980

Seminar, University of North Carolina, Chapel Hill, N.C., February 19-20, 1980

Conference to determine National Strategy for Radiation Research,
Washington, D.C., March 10-11, 1980

Seminar, Gray Laboratory, Northwood, England, April 16, 1980

British Institute of Radiology, Annual Congress, April 17, 1980

Seminar, Institute of Cancer Research, Sutton, England, April 18, 1980

Seminar, Methodist Hospital, Brooklyn, NY April 22, 1980

Seminar, Queens Hospital, Long Island, NY April 24, 1980

Science Writers Meeting, American College of Radiology, NY May 6, 1980

Seminar, Mallinckrodt Institute, St. Louis, MO May 8-9, 1980

High LET Workshop, NIH, Bethesda, MD May 16, 1980

Seminar, University of Tennessee, Memphis, TENN. May 22-23, 1980

Radiation Research Society Annual Meeting, New Orleans, LA. June 1-5, 1980

International Hyperthermia Conference, Fort Collins, Colorado June 22-26, 1980

Dr. Paul J. Kliauga

Annual Meeting, Radiation Research Society, New Orleans, LA, June 1-5, 1980

Dr. Richard C. Miller

CROS Radiosensitizer Meeting, Key Biscayne, Florida, October 3-6, 1980

Annual Meeting, Radiation Research Society, New Orleans, LA, June 1-5, 1980

International Hyperthermia Conference, Fort Collins, Colorado June 22-26, 1980

Dr. Harald H. Rossi

Press Conference, National Academy of Sciences, BEIR III Report, May 2, 1980

VIII International Conference on Atomic Collisions in Solids, Hamilton
Canada, August 13-17, 1980

Advances in Radiation Protection and Dosimetry in Medicine, Erice, Italy,
September 16-26, 1980

IEEE Nuclear Science Symposium, San Francisco, CA October 17-19, 1980

Workshop, Institute for Energy Analysis, Oak Ridge, TENN. October 30-31, 1980

American Institute of Biological Sciences, Radiation Biology Advisory
Panel, November 6-8, 1980

American Nuclear Society Forum, San Francisco, CA November 13, 1980

Physics Colloquium, Cornell University, Ithaca, NY November 19-20, 1980

Seminar, Colorado State University, Fort Collins, Colorado March 24-25, 1980

Colloquium, BEVALAC Microdosimetry Program, Lawrence Berkeley Laboratory, CA
March 27, 1980

Annual Meeting, NCRP, National Academy of Sciences, Washington, D.C.
April 2-3, 1980

Westinghouse Corporation - Radiation Information Tours, Michigan, April 6-9;
Missouri, May 12-14, 1980

Citizens for Safe Nuclear Energy, NY, NY May 21, 1980

Annual Meeting Radiation Research Society, New Orleans, LA June 1-5, 1980

Ms. Laurie Roizin-Towle

CROS Radiosensitizer Meeting, Key Biscayne, Florida October 3-6, 1980

Annual Meeting, Radiation Research Society, New Orleans, LA June 1-5, 1980

International Hyperthermia Conference, Fort Collins, CO June 22-26, 1980

Dr. Marco Zaider

Annual Meeting Radiation Research Society, New Orleans, LA June 1-5, 1980

THE COLUMBIA COLLOQUIUM

Each month during the year, regular colloquiums have been held to discuss ongoing research. These have been attended by the professional staff, graduate students, and senior technical staff of this laboratory, as well as scientists from the Safety and Environmental Protection Division of Brookhaven National Laboratory who are responsible for the running of RARAF, and scientists from other departments of the College of Physicians and Surgeons interested in collaborative research. Attention has focussed on recent findings, and future plans, with special emphasis on the interdisciplinary nature of our research effort.

During the year we have been pleased to welcome a number of visitors who have presented formal seminars and/or spent time discussing ongoing research with various members of the laboratory. These have included:

Dr. Charles Waldren
Eleanor Roosevelt Cancer Inst.
Denver, Colorado

Dr. G.E. Adams
Gray Laboratory
Northwood, Middlesex, U.K.

Dr. Michael Moore
Paterson Laboratories
Manchester, U.K.

Dr. John Hopewell
Churchill Hospital
Oxford, U.K.

Dr. Ian Stratford
Institute for Cancer Research
Sutton, U.K.

Dr. Paul Workman
MRC Unit
Cambridge, U.K.

Dr. James Belli
Harvard Medical School
Boston, Massachusetts

Dr. Evan Douple
Dartmouth University
Hanover, New Hampshire

Dr. John Leith
Brown University
Providence, Rhode Island

Dr. Joel Bedford
Colorado State University
Fort Collins, Colorado

Dr. Leo Sachs
Weizmann Institute of Science
Rehovoth, Israel

Dr. John Cleaver
University of California
San Francisco, California

OTHER ACTIVITIES

Members of the Laboratory were active on the following:

Dr. Marion Biavati

Sigma Xi

Dr. Richard P. Bird

Radiation Research Society

Dr. Carmia Borek

Assoc. Editor "Cancer Research"

Assoc. Editor J. of Environmental Pathology & Toxicology

Chairman & organizer of New York Academy of Sciences Conference on

Differentiation and Carcinogenesis in Liver cells in culture, Oct. 1979

Member, New York Academy of Sciences Conference Organizing Committee

Aging Review Committee (Program project review section) National Inst.
of Aging

Council Tissue Culture Association

Organizing Committee for Nato Conference on "The Use of Human Cells for
Risk Assessment by Physical and Chemical Agents"

Awards and Honors

Fellow of the New York Academy of Sciences

Dr. Michael Freeman

Radiation Research Society

Dr. Paul Furcinitti

Radiation Research Society

Dr. Charles R. Geard

Radiation Research Society

Dr. Paul Goldhagen

Radiation Research Society

Mr. Leon Goodman

Radiation Research Society

Dr. Eric J. Hall

Association for Radiation Research
British Institute of Radiology
Hospital Physicists Association
Oxford Society
Radiation Research Society
Sigma Xi

Committees

Radiobiology Committee RTOG (Chairman)
Radiation Research Society, Councilor
Editorial Board, Radiation Research
Editorial Board, Int. J. Radiat. Oncol. Biol. Phys.
American Board of Radiology, Therapeutic Radiology Test Committee
NCRP Committee 40
Committee for Radiation Oncology Studies

Dr. Paul J. Kliauga

ICRU Report Committee on Microdosimetry, member
Radiation Research Society
American Assoc. for the Advancement of Science
American Physical Society

Dr. Richard C. Miller

Radiation Research Society
Sigma Xi

Dr. Harald H. Rossi

International Commission on Radiation Units and Measurements (ICRU)

Main Commission, member

Critical Reviewer - Reports in Preparation:

Radiation Dosimetry: Electron Beams with energies between 1 and 50 MeV
High energy electron beam dosimetry

Technical Areas:

Radiobiology (Co-sponsor)

Radiation Physics - Neutrons and heavy particles (Co-sponsor)

Report Committees:

Clinical Dosimetry for Neutrons (Co-sponsor)

Fundamental Quantities and Units (member)

Microdosimetry (Co-sponsor)

National Council on Radiation Protection and Measurements (NCRP)

Main Council, member

SC-1: Basic Radiation Protection Criteria

SC-4: Heavy particles (Neutrons, Protons, and Heavier) (Chairperson)

SC-52: Conceptual Basis of Calculations of Dose Distributions (Chairperson)

Technical Advisory Committee on Radiation for the Commissioner of Health,
City of New York (Chairperson)
Nuclear Regulatory Commission (Consultant)
Tennessee Valley Authority (Consultant)
Committee on the Biological Effects of Ionizing Radiations (BEIR)
National Research Council
Awards and Honors Committee, Radiation Research Society
Co-Editor, Radiation and Environmental Biophysics
Ad Hoc Group for Technical Review, Div. Cancer Control and Rehabilitation,
National Cancer Institute

Societies

American Association for the Advancement of Science
American College of Radiology
American Radium Society
Sigme Xi, Kappa Chapter
Radiation Research Society
Radiological Society of North America

Ms. Laurie Roizin-Towle

Radiation Research Society
New York Academy of Sciences, full member

Dr. Marco Zaider

Radiation Research Society

BOOKS

"Differentiation and Carcinogenesis in Liver Cell Cultures" C. Borek and
G. Williams (eds.) Annals N.Y. Academy of Sciences, 1980.

Aging, Cancer and Membranes. C. Borek, C.M. Genoglio and D.W. King (eds.)
Stratton-Thieman Publishing Co., NY 1980.

"Biophysical Aspects of Radiation Carcinogenesis" A.M. Kellerer and H.H. Rossi
CANCER. A COMPREHENSIVE TREATISE. Vol 1. Etiology, 2nd Edition, Plenum
Publishing Corp, (in press).

"The Radiobiological Basis for Low Dose-Rate Brachytherapy", E.J. Hall,
in Modern Interstitial and intracavitary Radiation Cancer Management,
F. Genge (ed), Masson Publishing Inc., NY (in press).

BIBLIOGRAPHY

- Bird, R.P., Rohrig, N., Colvett, R.D., Geard, C.R., and S.A. Marino. Inactivation of Synchronized Chinese Hamster V79 Cells with Charged-Particle Track Segments, *Rad. Res.* 82: 277-289 (1980).
- Borek, C. Scanning Electron Microscopy of Differentiated Liver Cells Transformed *In Vitro* by Chemical Carcinogens. *J. Supramolecular Structure* 12: 293-298 (1979).
- Borek, C. X-ray Induced *In Vitro* Neoplastic Transformation of Human Diploid Cells. *Nature* 283(5749): 776-778 (1980).
- Freeman, M.L. The Transient Nature of Thermotolerance. *J. Natl. Can. Inst.* (in press).
- Geard, C.R. Effects of 1.9 MeV Monoenergetic Neutrons on *Vicia faba* Chromosomes: Microdosimetric Considerations. *Radiation and Environmental Biophysics* (in press).
- Geard, C.R. and M.H. Rutledge-Freeman. Effects of Long Contact Times of Misonidazole on Attached Chinese Hamster V-79 Cells. *Mutation Research* (in press).
- Goodman, L.J. Evaluation of Dose Equivalent Using a Tissue-Equivalent Ionization Chamber and a Geiger-Muller Dosimeter. *Rad. and Environm. Biophys.* 16: 367-371 (1979).
- Goodman, L.J. Uncertainties and their Treatment in Mixed-Field Dosimetry. Presented at the Workshop on Ionization Chambers for Neutron Dosimetry, Radiobiological Inst. TNO, Rijswijk, The Netherlands, Dept. 17-19, 1979.
- Goodman, L.J. and J.J. Coyne. W and Neutron Kerma for Methane-Based Tissue-Equivalent Gas. *Rad. Res.* 82: 13-26 (1980).
- Hall, E.J. Bleomycin: Drug Summary. *Int. J. Radiation Oncol. Biol. Phys.* 5: 1537-1539 (1979).
- Hall, E.J. Hyperthermia - An Overview. *J. Natl. Can. Inst.* (in press).
- Kellerer, A.M., Lam, Y.M.P. and H.H. Rossi. Biophysical Studies with Spatially Correlated Ions, IV. Analysis of Cell Survival Data for Diatomic Deuterium. *Rad. Res.* (in press).
- Miller, R.C. and E.J. Hall. Oncogenic Transformation *In Vitro* Produced by Misonidazole. *Cancer Clin. Trials* 3: 85-90 (1980).
- Miller, R.C., Hall, E.J. and H.H. Rossi. Oncogenic Transformation of Mammalian Cells *In Vitro* with Split Doses of X-rays. *Proc. Natl. Acad. Sci.* 76(11): 5755-5758 (1979).

- Miller, R.C., Harisiadis, L., Hall, E.J. and A. Napholz. Oncogenic Transformation *In Vitro*: The Interaction of X-rays with Hyperthermia. J. Natl. Cancer Inst. (in press).
- Mills, R. and H.H. Rossi. Mean energy deposition distribution about proton tracks. Rad. Res. (in press).
- Roizin-Towle, L., Hall, E.J. and L. Capuano. The Interaction of Hyperthermia and Cytotoxic Agents. J. Natl. Cancer Inst. (in press).
- Rossi, H.H. Biophysical Mechanisms of Radiogenic Cancer, in Radiation Biology in Cancer Research. R.E. Meyn and H.R. Withers (eds.) Raven Press, NY (1980).
- Rossi, H.H. Estimates of the Hazard of Low Doses of Ionizing Radiation. IEEE Transactions on Nuclear Science, NS-27(1): 23-27 (1980).
- Rossi, H.H. Microdosimetry and Its Application to Biology, in Radiation Protection and Dosimetry in Medicine, A Volume in Ettore Majorana-International Science Series, Plenum Publishing Corporation (in press).
- Rossi, H.H. The Molecular Ion Experiment. Nuclear Instruments and Methods 170: 57-59 (1980).
- Rossi, H.H. The Role of Microdosimetry in Radiobiology. Radiat. Environ. Biophys. 17: 29-40 (1979).
- Rossi, H.H. The Role of the Theory of Dual Radiation Action in Radiation Protection, in Radiation Protection and Dosimetry in Medicine, A Volume in Ettore Majorana-International Science Series, Plenum Publishing Corporation (in press).
- Rossi, H.H. The RBE of Neutrons. Proc. of Conference on Neutrons from Electron Medical Accelerators, National Bureau of Standards-SP 554: 37-39 (1979).
- Zaider, M. and H.H. Rossi. Synergistic Effects of Different Radiations. Rad. Res. (in press).