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# Oncogenic transformation in C3H10T½ cells by low-energy neutrons

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**Abstract.**
*Purpose*: Occupational exposure to neutrons typically includes significant doses of low-energy neutrons, with energies below 100 keV. In addition, the normal-tissue dose from boron neutron capture therapy will largely be from low-energy neutrons. Microdosimetric theory predicts decreasing biological effectiveness for neutrons with energies below about 350 keV compared with that for higher-energy neutrons; based on such considerations, and limited biological data, the current radiation weighting factor (quality factor) for neutrons with energies from 10 keV to 100 keV is less than that for higher-energy neutrons. By contrast, some reports have suggested that the biological effectiveness of low-energy neutrons is similar to that of fast neutrons. The purpose of the current work is to assess the relative biological effectiveness of low-energy neutrons for an endpoint of relevance to carcinogenesis: *in vitro* oncogenic transformation.
*Methods*: Oncogenic transformation induction frequencies were determined for C3H10T½ cells exposed to two low-energy neutron beams, respectively, with dose-averaged energies of 40 and 70 keV, and the results were compared with those for higher-energy neutrons and X-rays.
*Results*: These results for oncogenic transformation provide evidence for a significant decrease in biological effectiveness for 40 keV neutrons compared with 350 keV neutrons. The 70 keV neutrons were intermediate in effectiveness between the 70 and 350 keV beams.
*Conclusions*: A decrease in biological effectiveness for low-energy neutrons is in agreement with most (but not all) earlier biological studies, as well as microdosimetric considerations. The results for oncogenic transformation were consistent with the currently recommended decreased values for low-energy neutron radiation weighting factors compared with fast neutrons.

## 1. Introduction

For a variety of reasons, a significant number of individuals are occupationally exposed to low doses of neutrons, most of which are of low energy. For example, in US DOE facilities during 1988, about 92 000 individuals were monitored as potentially receiving neutron doses, and about 7000 individuals absorbed measurable neutron doses (Merwin *et al.* 1990). In addition, of the approximately 600 000 monitored workers under NRC regulation, about 6000 per year (primarily research workers, well loggers and reactor workers) receive measurable neutron doses (NRC 1988).

The neutron energy spectrum to which such individuals will be exposed varies widely, depending on the neutron source and the degree of shielding—and thus moderation—undergone by the neutrons, as well as neutron moderation in the body. Whether these effects are important depends on the variation in the relative biological effectiveness (RBE) of neutrons over the neutron energy range of interest for occupational and other exposures.

The significant neutron energy range, in terms of dose deposited, varies according to the fluence spectrum to which the individual is exposed. A typical example is presented in figure 1, showing kerma-weighted fluences as a function of neutron energy for a position where personnel are potentially exposed in a commercial nuclear reactor. For such situations,

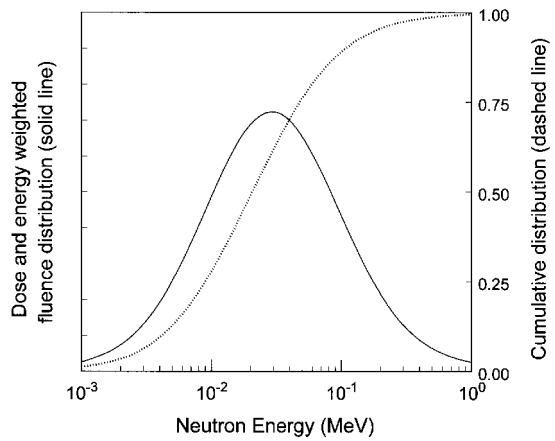


Figure 1. Distribution of neutron energies responsible for the deposition of neutron dose, for a typical location in a commercial pressurized water reactor where personnel might be exposed (Endres *et al.* 1981). The representation of the spectrum is such that equal areas under the full curve correspond to equal depositions of dose. The dashed curve is a cumulative representation of the same data, showing in this case, for example, that ~80% of the dose is deposited at energies below 50 keV.

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the important neutron energy range in terms of dose deposition is, on average, from about 10–200 keV (Endres *et al.* 1981).

The radiation weighting factors ($w_R$) currently recommended by the International Commission on Radiological Protection are 5 for neutrons of energy less than 10 keV, 10 for neutrons from 10 keV to 100 keV, and 20 for neutrons from 100 keV to 2 MeV (ICRP 1991). The data on which the radiation weighting factors for low-energy neutrons are based are, however, quite limited. For example, for the endpoint of chromosome aberrations, in the neutron energy range below ~100 keV, there are two major groups of data sets available, both based on filtered reactor beams: one is in the former Soviet Union (Sevankaev *et al.* 1979; nominal energy 40 and 90 keV), and the other in the UK (Lloyd *et al.* 1988, Morgan *et al.* 1988; nominal energy 24 keV). The yield (per unit dose at low doses) of chromosomal aberrations in human lymphocytes, as measured by Sevankaev *et al.* (1979), was considerably lower than the yield at neutron energies of a few hundred keV (see also NCRP 1990, table 2.9).

This measured decrease in RBE with decreasing neutron energy is in accord with earlier results for cellular survival and other biological endpoints (Hall *et al.* 1973, 1975, Underbrink and Sparrow 1974), and is also in accord with biophysical expectations (Kellerer and Rossi 1972, ICRU 1986), as well as recent ICRP recommendations (ICRP 1991). Microdosimetric considerations predict this decreased RBE with decreasing energy because, as illustrated in figure 2, the average energy deposited in a given cell (i.e. the average lineal energy) due to a neutron traversal decreases rapidly with neutron

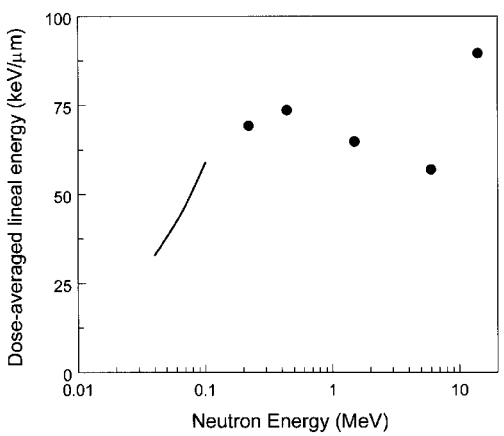


Figure 2. Measured and calculated dose-averaged lineal energies deposited by monoenergetic neutrons in 1 μm tissue-equivalent sites. Points above 200 keV represent experimentally measured data (Srdoc and Marino 1996). Curve below 200 keV shows calculated results at lower energies.

energy below about 350 keV (ICRU 1983, Srdoc and Marino 1996). This is both because of the decreasing LET of the recoil protons (the maximum proton LET is at a proton energy of about 100 keV) and the decreasing range of the recoil protons. For example, the range in tissue of a 20 keV proton (the average initial recoil proton energy produced by a 40 keV neutron) is about 0.5 μm, much less than a nuclear diameter.

In contrast with these measurements, the results of the Harwell group for chromosomal aberration yields induced by 24 keV neutrons in human lymphocytes, and for other endpoints in rodent cells (Lloyd *et al.* 1988), suggest yields comparable with those at a few hundred keV. This is significant at two levels. First, in terms of the radiation protection issues discussed above, a significant decrease in the biological effectiveness of neutrons in the energy range from the hundreds of keV to the tens of keV should result in a decrease in the radiation-weighting factor appropriate for most occupational exposure situations. Secondly, as has been discussed in terms of biological mechanisms, radiobiological models based on energy deposition in cellular or nucleus-sized targets (Kellerer and Rossi 1972, ICRU 1983, 1986) unequivocally predict a decrease in biological effect as the neutron energy decreases; if this decrease were not to be confirmed, then such models would be substantially falsified.

A final area of interest relates to the fact that low-energy neutrons are central to boron neutron capture therapy (BNCT). Here, low-energy neutrons are aimed at a tumour containing a borated drug, and neutron capture by a boron nucleus causes the emission of a highly damaging α-particle in the tumour. The limiting normal tissue damage may well be produced by the low-energy neutrons themselves.

To address the issue of the radiobiological effectiveness of low-energy neutrons, the Radiological Research, Accelerator Facility (RARAF) Van de Graaff accelerator was used to expose C3H10T½ cells to two low-energy neutron beams with dose-averaged mean energies of 40 keV and 70 keV. Previous studies from this laboratory have assessed the biological effectiveness of essentially monoenergetic neutrons in the energy range 200 keV to 15 MeV. Biological endpoints included cell survival in rodent cells (Hall *et al.* 1973, 1975), mutation in human–hamster hybrid cells (Hei *et al.* 1988), chromosomal aberrations in normal human cells (Pandita and Geard 1996), and oncogenic transformation in rodent cells (Miller *et al.* 1989). For all these endpoints, neutrons with energies between 350 and 450 keV showed a greater biological effectiveness than higher or lower-energy neutrons.

## 2. Materials and methods

### 2.1. *Irradiation procedure*

Protons from the RARAF Van de Graaff accelerator were used to produce low-energy neutron spectra by means of the $^{3}H(p, n)^{3}He$ reaction. Two different low-energy neutron spectra were used in this experiment, with maximum neutron energies respectively of 110 keV and 65 keV. The dose-weighted average neutron energies for the two spectra were approximately 70 and 40 keV, respectively. The contribution to the total dose from $\gamma$-rays was 3–6% in each case, as measured with a compensated Geiger–Muller detector.

The neutron-production target consists of tritium absorbed in a thin ($\sim 4\,\mu$m) layer of titanium plated on a flattened piece of copper tubing through which coolant water is flowed. The beam tube, target structure, and water lines have been minimized in size to allow samples to be placed in close proximity to the beam at any angle, and to reduce neutron scattering.

Samples were placed at an angle of 100° with respect to the proton beam direction, and at a distance of 25.4 mm from the centre of the target beam spot. Proton beams with energies of 1.3 and 1.4 MeV respectively were used to produce the 40 keV and the 70 keV spectra. The neutron production target was sufficiently thick to slow the protons below 1.15 MeV, the energy at which no neutrons are emitted at angles above 90° (the back threshold). Kinematic calculations, based on the cross sections, indicate that the initial neutron fluence spectrum is approximately triangular in shape, with the number of neutrons increasing with neutron energy up to the maximum. Calculated kerma-weighted fluence spectra for the two beams are shown in figure 3.

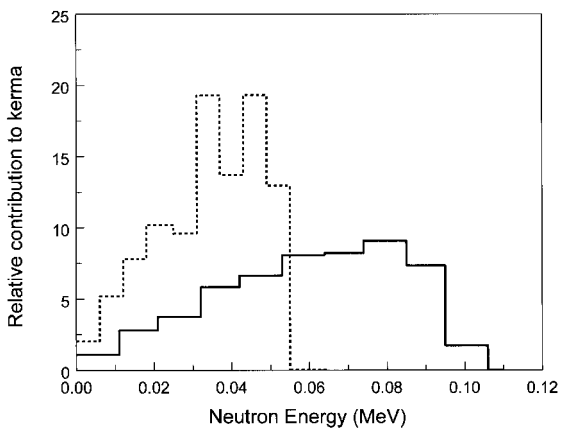


Figure 3. Calculated kerma-weighted fluence distributions for the two low-energy neutron beams under study. Solid line: 70 keV (dose weighted average) spectrum. Dashed line: 40 keV spectrum.

For comparison with the effects of higher-energy neutrons, cells were exposed to monoenergetic 350 keV neutrons, generated using the $^{3}H(p, n)$ reaction as described previously (Miller *et al.* 1989). In addition, cells under identical treatment conditions (pelletized in 1 mm pipettes) were exposed to 250 kVp X-rays (0.2 mm Cu and 1 mm Al external filtration) with a dose rate of 0.78 Gy/min.

Measurements of the total dose to ICRU muscle tissue at the location of the cells were made using a multiplication ionization chamber made of A-150 tissue-equivalent (TE) plastic and filled with standard methane-based TE gas. The chamber has a 6.4 mm diameter spherical sensitive volume and was positioned so that the centre of the volume was in the same location as the centres of the cell samples. A $^{226}Ra$ source originally calibrated by the National Bureau of Standards (NBS) was used to calibrate the chamber and a correction factor was applied for the mean energy to produce an ion pair ($W$ value) for neutrons relative to $\gamma$-rays. Corrections were applied to the dose measurements for attenuation by the sample, relative neutron kerma, and mass stopping power. Gamma-ray dose was measured using a similar ionization chamber with an aluminum wall and filled with argon.

The dose-averaged lineal energy (energy deposited due to neutron traversals in a 1 $\mu$m target—the microdosimetric correlate of LET (ICRU 1983)) for the beams has been measured for the X-ray beam and the 350 keV neutron beam, and was calculated for the two lower-energy neutron beams. The values were 4 keV/$\mu$m (X-rays), 70 keV/$\mu$m (350 keV monoenergetic neutrons), 47 keV/$\mu$m (70 keV neutron beam) and 33 keV/$\mu$m (40 keV neutron beam).

### 2.2. *Cell culture and preparation for irradiation*

Exponentially growing mouse C3H10T½ clone 8 cells of fibroblast origin between passages 9 and 15 were used in these experiments. Cells were grown in Eagle's basal medium with 10% heat-inactivated calf serum supplemented with iron (CBS-iron) from Hyclone Labs Inc., and 25 $\mu$g/ml gentamycin.

In order to minimize the dose and energy spreads of the low-energy neutrons to which the cells were exposed, the cells were concentrated into small pellets at the bottom of 1 ml plastic pipettes. The pipettes were modified by breaking off the lower two-thirds and heat-sealing the broken ends. Precise location of the cell pellets required heat-sealed pipettes that had a plastic plug 3 mm in diameter and exactly 1 mm high. Twenty minutes before treatment, the upper 50 mm of each pipette was snapped off and $2 \times 10^{5}$ cells in 0.2 ml of culture medium, purged with 5%

$CO_2$–95% air, were added. The opening of the pipette was sealed and cells were centrifuged at 1200 rpm for 5 min to pack cells towards the plastic plug at the bottom of the pipette.

Immediately after irradiation, cells were trypsinized and replated into 100 mm diameter tissue culture dishes at cell concentrations estimated to result in either 300 clonogenic cells per dish (for the transformation assay) or 30 clonogenic cells (for the cell survival assay). Cell densities for the transformation studies varied from 220 to 310 clonogenic cells per dish. At the end of the incubation period, cells were fixed in formalin and stained with Giemsa. Cell survival was determined by the colony assay method, and transformed foci types II and III were identified according to criteria described by Reznikoff *et al.* (1973a,b) and IARC (1985).

Most dose points were repeated in three to four replicate experiments, each replicate experiment also including its own zero dose controls. The total number of dishes exposed for the oncogenic transformation experiments was about 1100 for the low-energy neutron experiments, about 1300 for the fast neutron experiments and about 2000 for the X-ray experiments, with a total of about 1100 control (sham-irradiated) dishes.

## 3. Results

Initial studies were performed to determine whether pelletization induced cellular perturbations that would have a significant effect on survival or the appearance of morphological transformants. Cells either in conventional exposure conditions (Pandita and Geard 1996) consisting of attachment to the growth surface of Falcon 25 $cm^2$ culture flasks or in pellets at the bottom of the modified pipettes were exposed to either X-rays or to 350 keV monoenergetic neutrons. The plating efficiency as a result of cell pelletization was somewhat more variable (varying from 23% to 35%) than for cells plated in culture flasks, but the induced rates of survival and transformation were indistinguishable regardless of the exposure conditions of cells.

Results for cells exposed to neutron beams with average energies of either 40 or 70 keV, as well as those for 350 keV monoenergetic neutrons and for X-rays, are shown in figure 4. The measured transformation rates per surviving cell were fitted as functions of dose, $D$, to the linear–quadratic expression:

$$R_i = b + \alpha_i D + \beta D^2, \qquad (1)$$

where the subscript $i$ ($i = 1, 4$) refers to the radiation type. Note that a single, common value of the quadratic parameter, $\beta$, was fitted for all the radiation types. The parameter $b$ refers to the background (zero dose) transformation rate, which was measured separately in each replicate experiment (each dose point corresponded with three to four replicate experiments, each with its own internal zero-dose control). The estimated values of the parameter $b$ were separately assessed in the fitting procedure for each replicate experiment. The curve fitting and parameter estimation were performed using standard maximum likelihood algorithms (specifically the iteratively-reweighted non-linear least squares technique (Press *et al.* 1986)), with the quoted uncertainties in the parameters being assessed by the technique of synthetic data simulation (Press *et al.* 1986). Based on the Pearson $\chi^2$ test, equation 1 provides an adequate fit to the data.

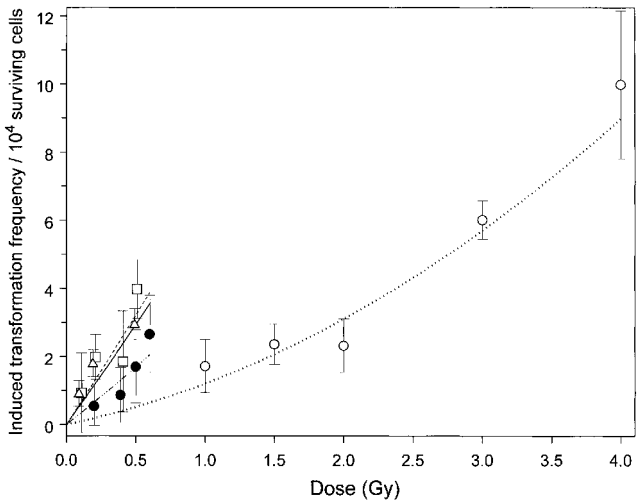


Figure 4. Induced oncogenic transformation frequency (with 95% confidence limits) as a function of radiation dose for C3H10T½ cells exposed to 350 keV monoenergetic neutrons (△, dashed curve), neutrons with a dose-averaged energy of 70 keV (□, solid curve) or 40 keV (●, dot-dash curve) and 250 kVp X-rays (○, dotted curve); curves refer to fits of the data to equation 1, with controls (the parameter $b$ in equation 1) subtracted out.

The estimated $\alpha$ values (the low-dose biological effectiveness) from the fit of the data to equation 1 are shown in table 1. When the dose-averaged neutron energy is decreased from 350 keV to 70 keV to 40 keV, there is a corresponding decrease in the point estimates of $\alpha$, the low-dose biological effectiveness. For example, the low-dose effectiveness of the 40 keV neutrons is approximately half that of the maximally effective (Pandita and Geard 1996) 350 keV neutrons.

In order to inter-compare directly the results from the four different radiation types (three neutron energies and the X-ray beam), for each of the six

Table 1. Estimated linear dose–response parameters and corresponding maximal (low-dose) RBE values for oncogenic transformation induced by different radiations.

| Radiation | $\alpha_i$ ($Gy^{-1}$, see eq. 1) | $RBE_{max}$ |
|---|---|---|
| X-rays | $0.87 \pm 0.39$ | – |
| 40 keV neutrons | $3.22 \pm 0.97$ | $3.7 \pm 1.9$ |
| 70 keV neutrons | $5.7 \pm 1.1$ | $6.6 \pm 3.1$ |
| 350 keV monoenergetic neutrons | $6.26 \pm 0.61$ | $7.2 \pm 3.3$ |

Standard errors are also shown.

The corresponding (common) value of the quadratic ($\beta$) term (see equation 1) was $0.35 \pm 0.10\ Gy^{-2}$.

The raw data are available from the authors, on request.

pairs of radiation types, the data for that pair were fitted both to equation 1 (with $i = 1, 2$), and also fitted by equation 1 using a single common value of $\alpha$ for both radiations. For each pair of radiation types, the residuals from the individual-$\alpha$ and the common-$\alpha$ fits were then compared using an *F*-test to examine the null hypothesis that the data from both radiation types came from the same distribution. The results, shown in table 2, indicate that the 40 keV neutron beam was significantly less effective than 350 keV neutrons. The 70 keV neutrons were intermediate in effectiveness between the 40 keV and the 350 keV neutrons, though not statistically different from either, at the 95% confidence level.

The maximal (low-dose) RBE of the different neutron beams relative to X-rays was calculated for each of the neutron energies under study using the relation, $RBE_{max} = \alpha_n / \alpha_x$. These calculated RBE values are also shown in table 1.

Doses chosen in the experimental design were such that generation of RBE values for oncogenic transformation was optimized. Comparable RBE values were estimated for the endpoint of cell killing, but the standard errors were large due to the X-ray doses chosen. The RBE for cell killing with the 70 keV and 40 keV neutrons relative to 350 keV neutrons were respectively $0.6 \pm 0.2$ and $0.7 \pm 0.2$, which are consistent with the values for oncogenic transformation.

Table 2. Results of pairwise comparison between radiations.[a]

| Radiation | X-rays | 40 keV neutrons | 70 keV neutrons |
|---|---|---|---|
| 350 keV neutrons | *S* | *S* | *NS* |
| 40 keV neutrons | *S* | – | *NS* |
| 70 keV neutrons | *S* | *NS* | – |

[a]Results derive from pairwise *F*-tests (95% confidence level), comparing fits to data for pairs of radiations using the models $R_i = b + \alpha_i D + \beta D^2$ ($i = 1, 2$), and the smaller model $R = b + \alpha D + \beta D^2$ (i.e. with the same $\alpha$ parameters for both radiations).

*NS* means that the smaller model cannot be rejected, implying that there is not statistically significant evidence that the effects produced by the two radiations come from different distributions.

*S* means that the smaller model can be rejected, implying that it is likely that the effects produced by the two radiations come from different distributions. Thus, for example, the middle column implies that the 40 keV neutrons were significantly less effective than the 350 keV neutrons, but not significantly different in effectiveness compared with the 70 keV neutrons.

## 4. Discussion

In radiation protection practice, the relative risks associated with exposure to low doses of various ionizing radiations are compared quantitatively by multiplying the absorbed dose with the radiation weighting factor $w_R$; this weighting factor is evaluated based on biological experiments, where possible, or from theoretical predictions when there is insufficient biological data.

Based on the microdosimetric considerations (Kellerer and Rossi 1972), below about 350 keV the RBE of neutrons is predicted to decrease with decreasing energy. This is because the average energy deposited in a given cell due to a neutron traversal decreases rapidly with neutron energy, below about 350 keV (ICRU 1983), both because of the decreasing LET and the decreasing range of the recoil protons. In fact, the dose average lineal energy deposited in 1 $\mu$m sites by the 40 keV neutron beam in this study was only about half of that from the 350 keV neutron beam.

Biological validation of this predicted decrease in RBE with decreasing neutron energy has come from a variety of investigators, radiation sources and biological model systems. In the USA, several investigators have studied a variety of biological endpoints with the same two low-energy neutron beams as used in the current study. Underbrink and Sparrow (1974) showed that the RBE for *Tradescantia occidentalis* stamen hair mutations was significantly less for neutrons with a dose-averaged energy of ~40 keV than for ~70 keV dose-averaged-energy neutrons. Hall *et al.* (1973) described the inhibition of root growth in *Vicia faba* seedlings after exposure to low-energy neutrons and reported lower RBE values for a spectrum of neutrons with a mean energy of 40 keV than for a higher energy neutron spectrum with a dose-averaged mean of 70 keV. Similar results for cell survival after low-energy neutron exposures of V79 mammalian cells have also been reported (Hall *et al.* 1975). Sevankaev *et al.* (1979), in the Soviet Union, measured dicentric chromosomal aberration yields in human lymphocytes, and showed a similar reduction in RBE as the mean neutron energy was reduced from 90 to 40 keV.

On the other hand, a series of reports based on

studies at the Harwell PLUTO fission in the United Kingdom, with a filtered beam of (nominally) 24 keV neutrons, suggested that the RBE of keV neutrons was not less than the maximum value exhibited by higher-energy neutrons (Morgan *et al.* 1986, Roberts *et al.* 1987, Lloyd *et al.* 1988, Morgan *et al.* 1988, Edwards *et al.* 1990); these results were obtained both for cell survival and for chromosome aberration induction.

There remains then disagreement between, on the one hand, the results from the Harwell group (Morgan *et al.* 1986, Roberts *et al.* 1987, Lloyd *et al.* 1988, Morgan *et al.* 1988, Edwards *et al.* 1990) and, on the other hand, the current results, i.e. those of Sevankaev *et al.* (1979), Underbrink and Sparrow (1974), Hall *et al.* (1973, 1975), as well as microdosimetric predictions (Kellerer and Rossi 1972, ICRU 1983, 1986). The basis for this disagreement is not clear. One interpretation is that the Harwell group did not directly compare their results at 24 keV with 'maximally effective' ~ 350 keV neutrons but, rather, with fission neutrons (Lloyd *et al.* 1976); in fact, using the radiation-quality based model that the Harwell group derive to describe all their chromosome aberration data (Edwards *et al.* 1990), the experimental results from Edwards *et al.* (1990) for 24 keV neutrons are about 40% less than would be predicted in a maximally effective neutron beam. A second interpretation might be that the fast neutron contamination of the Harwell 24 keV beam (where higher-energy neutrons were estimated to contribute ~ 21% of the dose (Perks *et al.* 1988)) was underestimated and/or contributed significantly to the measured biological effectiveness.

In conclusion, the current study provides evidence for a decrease in RBE with decreasing neutron energy below about 350 keV for the endpoint of oncogenic transformation. This result is in agreement with most (but not all) earlier biological studies, as well as microdosimetric considerations. Therefore, it appears reasonable to maintain a smaller radiation weighting factor for neutrons with energies below 100 keV compared with those for higher-energy neutrons (ICRP 1991).

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