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The Biophysical Properties of 3.9-GeV Nitrogen Ions
III. OER and RBE Determinations Using *Vicia* Seedlings [1]

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HALL, E. J. AND KELLERER, A. M., The Biophysical Properties of 3.9 GeV Nitrogen Ions. III. OER and RBE Determinations Using *Vicia* Seedlings. *Radiat. Res.* 55, 422–430(1973).

Seedlings of *Vicia faba* were used as a test system to investigate the radiobiological properties of a beam of 3.9 GeV nitrogen ions. Samples were irradiated on the entrance plateau and in various positions close to the Bragg peak of the depth–dose distribution. The oxygen enhancement ratio (OER) was found to be 1.55–1.65 at the Bragg peak, but had a value indistinguishable from that of x-rays at all other positions in the beam. The relative biological effectiveness (RBE) is a function of dose, and therefore of the level of biological damage. For 50% growth inhibition the RBE is between 1 and 1.9 on the entrance plateau, and is about 6 at the Bragg peak. In the region a few millimeters upstream of the Bragg peak the RBE remains nearly constant in spite of a rapid variation of average LET. Over the same region the OER rises rapidly from the low value determined at the peak (1.55–1.65) to the value characteristic of conventional x-rays (2.5–3).

Until 1971 high energy heavy ions were unique to the space environment. During July of that year 3.9 GeV nitrogen ions were successfully accelerated, first at the Princeton Particle Accelerator, and soon afterward as the Berkeley Bevatron. At first the beam obtained was suitable only for physical measurements because the flux was too low; however, during the latter part of 1971 and the spring months of 1972 the particle flux was increased to a point where biological experiments were possible, and a limited amount of time was made available for such experiments before the machine closed down due to lack of funds.

Columbia University is situated in New York City, some 60 miles from the Princeton Accelerator. No laboratory facilities were available at the accelerator site and this factor, together with the limited beam time available, imposed severe restrictions upon the biological experiments which could be contemplated. Nevertheless, in view of the uniqueness of the opportunity, and the probability that many years will elapse before comparable high energy ions

[1] This investigation was supported by Contract AT-(11-1)-3243 from the U. S. Atomic Energy Commission and by Public Health Service Research Grant No. CA-12536.

are again available for research on the East coast, it was felt that a considerable experimental effort was justified in spite of the suboptimal conditions. The two previous papers in this series (*1, 2*) describe the physical measurements of absorbed dose and dose distribution, which provide a foundation for the biological research described in this and the following two papers.

The stimulus to perform biological measurements with 3.9 GeV nitrogen ions is twofold. In the first place, such measurements are of immediate concern in basic radiobiology, since the radial profile of energy deposition in tracks around the main core of ionization for these heavy particles is different from that for more conventional radiations such as x-rays or neutrons. The relationship between the pattern of energy deposition and the subsequent biological effect is of considerable interest in the interpretation of basic mechanisms of radiation damage.

In the second place, there is a possibility that these high energy heavy ions may have an application in radiotherapy. The combination of a favorable depth–dose pattern associated with the Bragg peak, together with the possibility of a lowered oxygen enhancement ratio (OER) resulting from the densely ionizing terminal portion of the track of the high energy particles, make a beam of heavy ions, at least in theory, an attractive possibility for radiotherapy. Heavy ions offer the possibility of delivering a high dose of radiation to a sharply delineated tumor volume, with a high radiobiological effectiveness and a low oxygen enhancement ratio, so that hypoxic tumor cells within the target volume would not be protected and spared by their deficiency of oxygen. At the same time, normal tissues traversed by the beam before it reaches the tumor would be subjected to a much lower dose, and in addition the radiation in this region would have a lower biological effectiveness.

Seedlings of *Vicia faba* are a classical radiobiological test system, and have been used for over 40 yr (*3, 4*). This present communication describes their use to investigate the oxygen enhancement ratio and the relative biological effectiveness at various points in the depth–dose curve for the nitrogen ion beam. They were chosen for this project for two reasons. First, these hardy plant seedlings may be subjected to vigorous gassing procedures with high purity nitrogen to render them hypoxic, without apparent damage. A satisfactory level of hypoxia is more readily produced and maintained with this biological material than with most others, particularly mammalian cells. Second, they were chosen because they are more sensitive to radiation than mammalian cells, and consequently require lower doses to produce a level of biological damage which may be scored. This was an overriding consideration in the early experiments in which the dose rate in the nitrogen ion beam was a limiting factor, but it ceased to be an important consideration in later experiments when the dose rate at the position of the Bragg peak exceeded 40 rads/min.

MATERIALS AND METHODS

Culture of the Seedlings

In each experiment about 500 seeds were soaked in running water for 3 days; those that had germinated were planted in moist vermiculite, a medium used

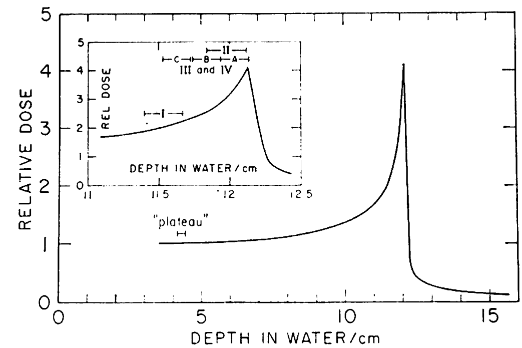

FIG. 1. Depth–dose distribution for a beam of 3.9 GeV nitrogen ions; showing the position at which seedlings were irradiated in the various experiments.

commercially to raise seedlings, where they were left for 5 days. By this time the primary root of each seedling was approximately 10 cm in length, at which time they were transferred to the main culture tank, and placed on a Lucite lid with their roots pointing downward into the culture medium maintained at 19° centigrade. The culture medium consists of a solution of salts and trace elements and has previously been described (*5*).

The seedlings were sorted into groups of nine, carefully matched so that the distribution of lengths within each group was similar. The seedlings to be irradiated, together with controls, were transported the 60 miles from Columbia to Princeton in Lucite containers which were insulated to minimize temperature fluctuations, and in which vigorous aeration was maintained at all times. The details of the culture procedure have previously been reported (*6*).

The irradiations were carried out in specially constructed Lucite fixtures, designed to accommodate nine seedlings in such a way that the root tips came together into a small volume, 1.25 cm square in cross section. In the early experiments this small volume in which the root tips were constrained was 3 mm deep in the direction of the beam, but for the later experiments this distance was further reduced to 2 mm, which constitutes the practical limit. The lid of each fixture was screwed down tightly onto a rubber O-ring to effect a seal. The seedlings were aerated or rendered hypoxic by bubbling a stream of air or high purity nitrogen at a flow rate of 1 liter per minute through the water into which the primary roots of the seedlings were dipped. In the latter case, the gas was flushed through the system for 30 min prior to irradiation, which was shown in previous experiments to be adequate to achieve an acceptable level of hypoxia (*7*).

Prior to irradiation the length of each primary root was measured from a reference point on the hypocotyl to the growing tip. Roots were cultured for a period of 10 days at 19° centigrade, and at the end of this time each root was remeasured and the growth increment during the 10 day period calculated. The mean 10-day increment for each group, divided by the corresponding quantity for control roots, is termed "growth in 10 days." This quantity is

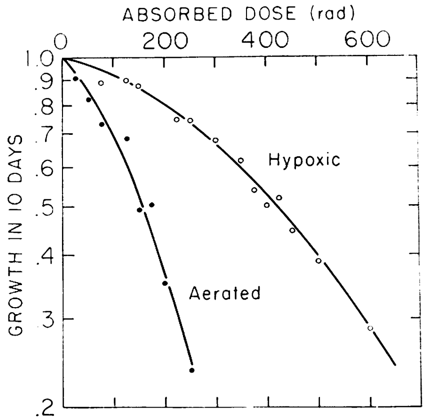

FIG. 2. Dose–effect curves for *Vicia* seedlings exposed to x-rays. The closed and open symbols refer to aerated and hypoxic conditions, respectively.

a measure of the radiation damage in the root meristem, and has been used for many years (*8*, *9*).

RESULTS

Four separate visits were made to Princeton, which are designated in the results by I to IV. The position in the beam in which measurements were made in the various experiments are indicated on the depth–dose curve in Fig. 1. Parallel experiments were performed with 210 kV x-rays at a dose-rate of 70 rads/min (added filtration: $\frac{1}{4}$ mm Cu + 1 mm Al).

The data obtained from the x-ray experiments are presented in Fig. 2, where the "growth in 10 days" (G/G_0) is plotted on a logarithmic scale agsinst dose (D), on a linear scale, for aerated and hypoxic conditions.

In all cases a least squares fit to the equation

$$G/G_0 = e^{-aD-bD^2} \tag{1}$$

has been performed and the 95% confidence region for the parameters G_0, a, and b has been derived. The linear regression analysis employed for this purpose is based on Fisher's F-distribution [see for example (*10*)]; it utilizes the mean growth increment for each irradiated group and weights each point according to the variance of growth observed in this group. Equation (1) has previously been used to represent the dose–response curve for x-rays (*5*). In this case the parameters $a = 2.45 \cdot 10^{-3}$ (rad^{-1}) and $b = 1.24 \cdot 10^{-5}$ (rad^{-2}) have been obtained for aerated conditions, while the estimates for hypoxic conditions are $a = 0.63 \cdot 10^{-3}$ (rad^{-1}) and $b = 0.25 \cdot 10^{-5}$ (rad^{-2}). The values for aerated conditions and x-rays will in the following be used to establish RBE values for nitrogen ions. The data for x-rays and the best fit to Eq. (1) are represented in Fig. 2.

For the data obtained with nitrogen ions it is in all cases found that the quadratic term in Eq. (1) does not significantly contribute to the optimum fit.

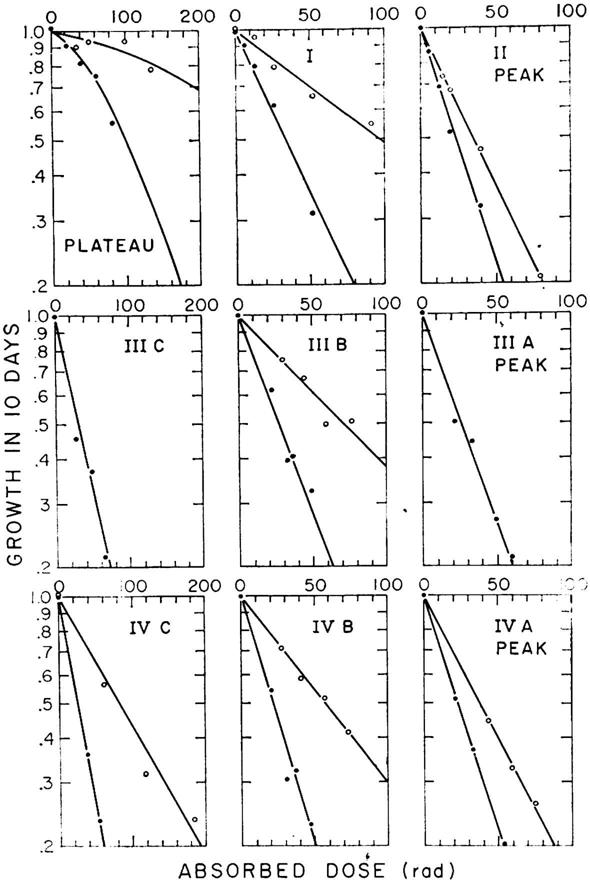

FIG. 3. Dose effect curves for *Vicia* seedlings exposed to 3.9 GeV nitrogen ions. The closed and open symbols refer to aerated and hypoxic conditions, respectively.

The parameter b can according to the regression analysis in all cases be equal to the value obtained for x-rays; but since its inclusion does not significantly affect the shape of the resulting curves Eq. (1) has been reduced to the simple exponential relation:

$$G/G_0 = e^{-aD}. \qquad (2)$$

The least square fits to this equation are represented in Fig. 3 and the estimates and 95% confidence limits for the coefficient a are given in Table I.

An exception has been made in Fig. 3 with the data obtained in the region of the initial plateau of the depth–dose curve. In this particular case the data are so close to those observed for x-rays that it appears unjustified to exclude the quadratic component in the reaction. For this reason the optimum fit to the linear-quadratic equation (1) has been inserted; one must, however, be aware that in this particular experiment the technical difficulties due to the severely limited dose rate (about 1 rad/min) lead to statistical variations

TABLE 1

SUMMARY OF RESULTS OF STATISTICAL ANALYSIS OF DATA

Position	*OER and 95% confidence limits*	*a (rad⁻¹); aerated and 95% confidence limits*	*a (rad⁻¹); hypoxic and 95% confidence limits*	*RBE₀*	*RBE₅₀ (aerated)*
Plateau	~3	$\sim 0.3 \cdot 10^{-2}$	$\sim 0.1 \cdot 10^{-2}$	1–1.9	1–1.9
I	2.5 (2–3.2)	$2\ (1.75–2.25) \cdot 10^{-2}$	$0.75\ (0.55–0.95) \cdot 10^{-2}$	8.2	4.5
II	1.55 (1.4–1.7)	$3\ (2.3–3.7) \cdot 10^{-2}$	$1.9\ (1.65–2.15) \cdot 10^{-2}$	12.3	6.8
III A	—	$2.6\ (2–3.2) \cdot 10^{-2}$	—	10.7	5.9
B	2.43 (1.8–3.3)	$2.5\ (1.6–3.4) \cdot 10^{-2}$	$1.1\ (0.7–1.5) \cdot 10^{-2}$	10.3	5.6
C	—	$1.95\ (1.3–2.6) \cdot 10^{-2}$	—	8	4.4
IV A	1.65 (1.56–1.73)	$2.9\ (2.2–3.6) \cdot 10^{-2}$	$1.8\ (1.3–2.3) \cdot 10^{-2}$	11.9	6.5
B	2.66 (2–3.5)	$3.3\ (1.8–4.8) \cdot 10^{-2}$	$1.2\ (1–1.4) \cdot 10^{-2}$	13.6	7.4
C	2.90 (2.6–3.4)	$2.6\ (2.1–3.1) \cdot 10^{-2}$	$0.8\ (0.5–1.1) \cdot 10^{-2}$	10.7	5.9

which are too large to allow statistically significant statements on the shape of the dose effect relation.

The estimated values of OER, and their confidence limits, are not obtained directly from the estimates and the confidence limits of the coefficients a in the oxygenated and hypoxic case. A direct derivation is not possible because in each experimental group the experiments in oxygenated and hypoxic condition share the same controls and because the confidence regions are accordingly not statistically independent. The OER must therefore be analyzed by a separate statistical test, and for this purpose the parallel line assay which has been used earlier (*5, 6*) has been applied. The resulting estimates and confidence ranges for the OER are also given in Table I.

In the first experiment, Princeton I, specimens were irradiated at two positions; on the initial plateau and in position 1, 3–6 mm upstream from the peak. The data obtained on the plateau are limited because the dose-rate (about 1 rad/min) was low in this position. All that can be said is that the radiobiological properties of the beam in this position differ little from those of x-rays. The statistical uncertainty of the value of OER is so large that no deviation from the value for x-rays can be inferred. The RBE for 50% growth reduction could be equal to 1 and is with 95% significance below 1.9. In the position just upstream of the peak the OER is near to 3. In the second experiment, Princeton II, the specimens were located as close to the peak as possible and the resultant data indicate an OER of approximately 1.55.

Experiments III and IV were both performed with the seedlings in the redesigned fixture with the root tips confined within a dimension of 2 mm in the direction of the beam. These two experiments were intended to be a repeat of one another, and great care was taken in the positioning of the samples on the two occasions. The OER at the position of the peak, position A in experiment Princeton IV, has a value of about 1.6, and is not significantly different from the value obtained in Exp. II. The OER obtained in Exp. IV, position C, 4 mm upstream from the peak is near 3 which again is a good repeat of the value obtained in the corresponding position in Exp. I.

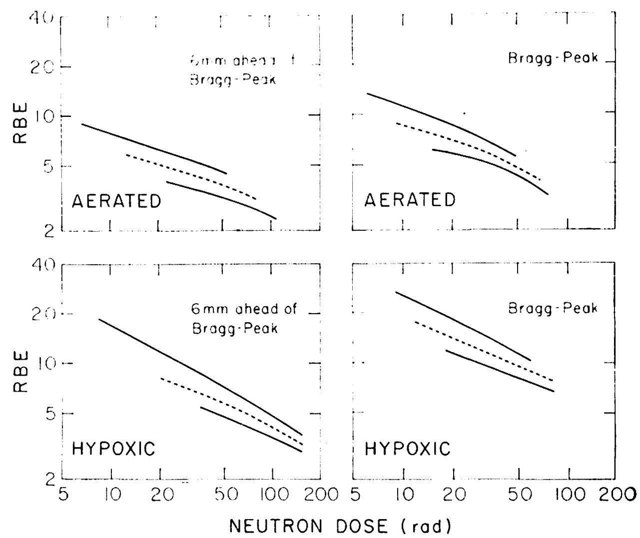

FIG. 4. Estimates and 95% confidence regions for the RBE of nitrogen ions at different positions in the particle beam.

Figure 2 shows corresponding data for an x-ray experiment; the OER in this case is approximately 2.7 which is consistent with the values obtained on many previous occasions.

The specification of RBE is complicated by the fact that the dose–response curves for nitrogen ions have different shapes than the dose–response curve for x-rays, and that accordingly RBE is a function of dose, or of the effect level. The extrapolated values, RBE_0, for smallest effects are equal to a_N/a_x where a_N and a_x are the estimated parameters in Eq. (1) and (2) for x-rays and nitrogen ions. With the value of $2.45 \cdot 10^{-3}$ one obtains the following relation between RBE_0 and the parameter a_N:

$$RBE_0 = 410 \cdot a_N. \tag{3}$$

These values are also included in Table I. The RBE for 50% growth reduction can be expressed in terms of a_N and the x-ray dose, $D_{x,50}$, for 50% growth reduction. Entering a_x and b_x in Eq. (1) one obtains $D_{x,50} = 156$ rad. Therefore one has:

$$RBE_{50} = \frac{D_{x,50}}{D_{N,50}} = \frac{156}{\ln 0.5} a_N = 225 a_N. \tag{4}$$

These values of the relative biological effectiveness for 50% growth reduction under oxygenated conditions are given in the last column of Table I.

The values of RBE discussed above are estimates based on the least square fit of the experimental data to Eqs. (1) and (2). In order to obtain confidence intervals for the RBE values a more direct statistical analysis has been applied. This analysis is based on a nonparametric test which compares the result of each dose of nitrogen ions with the result of each x-ray dose, and which uses the resulting statistical significance levels to derive the confidence region in the plane of RBE versus dose. The details of this statistical analysis will be given in a separate paper (*11*). The analysis can only be successfully applied to the data taken near the Bragg peak where the data have sufficient statistical accuracy. Figure 4 presents the results for Exp. I and for the pooled data of Expts III and IVA. The solid lines give the borders of the 95% confidence regions. The broken lines are the estimated curves for RBE. These lines do not significantly deviate from the lines of slope -0.5 which are predicted by the theory of dual radiation action (*12*).

DISCUSSION

On the initial plateau of the depth–dose curve, the data available are more limited than in the peak region and leave much to be desired. They are, however, adequate to indicate that in this region the enormously energetic heavy charged ions resemble conventional x-rays in their radiobiological properties. They exhibit an OER of 2.5–3, and the RBE is not much more than 1, certainly less than 1.9. In the region of the sharply defined Bragg peak, near the end of the range of the nitrogen ions, the RBE rises to a higher value.

In position 1, on the "upstream" side of the Bragg peak, the RBE (for the defined end point of 50% growth inhibition) is 4.3. At all other positions near the Bragg peak the RBE is in the range 5–6.8, and the variations are probably not significant. Over the region for which the RBE remains nearly constant, the average LET is varying very rapidly. The explanation for this observation is simple. As the terminal few millimeters of the range is approached, inefficient low LET components of the beam are lost, only to be replaced by equally inefficient, very high LET components (*13*).

While the RBE does not appear to vary appreciably over the few millimeters upstream of the Bragg peak, the same cannot be said for the oxygen enhancement ratio. A high value for the OER, indistinguishable from that characteristic of conventional x-rays, was found in all positions of the beam with the exception of the position right at the Bragg peak. Only in the very narrow region situated right at the Bragg peak was the oxygen enhancement ratio reduced. In the two experiments performed at the peak, values of 1.55 and 1.65 were obtained.

The implication of these results for basic radiobiology will be discussed in considerably more detail in the last paper of this series. As far as radiotherapy is concerned, they indicate that nitrogen ions will probably be unsuitable for clinical use. The Bragg peak is very narrow, which is not of itself a serious limitation because it could be broadened by the use of, for example, a ridge filter. However, the region over which the OER is low is very narrow indeed, and the average OER in a spread out Bragg peak is unlikely to offer sufficient advantage to warrant the trouble and expense of using this radiation modality. The general characteristics of the nitrogen beam do indicate strongly that a further study of high energy heavy ions is called for, and may well lead to exciting results. Particles of higher Z clearly need to be investigated because they would be expected to exhibit a lowered OER over a larger region of the terminal portion of their track; this characteristic would be ideal for applications in clinical radiotherapy.

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