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W. D. Loughman, J. M. Feola, M. R. Raju, and H. S. Winchell

April 12, 1967

RBE of π^- Beams in the Bragg Peak Region
Determined with Polyploidy Induction¹
in Mammalian Cells Irradiated In Vivo

W. D. Loughman, J. M. Feola, M. R. Raju, and H. S. Winchell²

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ABSTRACT

The RBE of components of a π^- -meson beam, contaminated with electrons and π^- decay products, was determined for polyploidy induction in a mammalian cell system in vivo. The RBE in the plateau region of this beam was 1 relative to cobalt-60 gamma rays, whereas in the Bragg-peak region it was 2.15. For pion effects only in the peak region, RBE was 2.37; for "star" effects only, it was 3.64. On the basis of theory and preliminary observation, it is believed that RBE values for cell killing exceed the values found for polyploidy induction. We conclude that the diminished oxygen effect and low ratio of surface dose to depth dose displayed by π^- beams having a Bragg peak region, coupled with the Bragg-peak region's relatively high RBE, indicate π^- beams may be useful in tumor radiotherapy.

KEY WORDS: RBE

Pi-Meson

Mammalian

Bragg Peak

INTRODUCTION

In the search for modes of radiotherapy for deep-seated tumors, attention has become increasingly centered on the potential usefulness of π^- -meson (hereafter called π^- for brevity) beams. The π^- has a finite range terminated by a Bragg peak. At the end of its range, it interacts with the nuclei of light elements, and the resulting nuclear fission adds to the energy deposition in the Bragg peak region. Fowler and Perkins (1), Fowler (2), Aceto,³ and Richman et al. (3)⁴ have observed the characteristics of π^- beams in physical systems. Their work collectively indicates π^- beams have a low ratio of surface dose to depth dose, a diminished oxygen-enhancement ratio, and a probable high relative biological effectiveness (RBE).

Artificially produced π^- beams have been available since 1948, but biological investigations with π^- were not performed until 1963. At that time, Micke et al. (4, 5) used the 7-9-GeV π^- meson beam from the Brookhaven alternating gradient synchrocyclotron to irradiate seeds of Zea mays. Despite the low dose rate of tenths of rads per minute, these workers obtained an RBE of 3.23 for non-Bragg peak π^- mesons.

The Berkeley 184-inch synchrocyclotron (with which we worked) produces π^- of 90-95 MeV at a dose rate of approximately 5 rads/hour. Loughman et al.^{5, 6} and Feola et al.^{7, 8} have used this beam for investigations of the Bragg-peak region on mammalian tumor cells in vivo. Richman et al.⁹ have used the same beam for similar studies on bean root meristem. In all these experiments, x-ray comparison data was not available, and RBE could not be determined.

We describe here the results of acute irradiation of mammalian tumor cells in vivo by (a) ^{60}Co gamma rays, (b) the "plateau" (minimum ionizing) region, and (c) the augmented Bragg-peak ("star") regions of a π^- beam. An estimate of the RBE of π^- for the induction of mammalian cell polyploidy is given.

MATERIALS AND METHODS

π^- -Beam Production

Full descriptions of the apparatus associated with the 184-inch synchrocyclotron at Berkeley, and of methods for producing the 90-MeV pion beam, have been given elsewhere (3).^{3, 4} This experimental arrangement is shown schematically in Fig. 1. Briefly, pions are produced by impinging 732-MeV protons on a beryllium target within the cyclotron. The cyclotron's magnetic field deflects pions and contaminating particles out of the cyclotron tank. A small quadrupole focusing magnet delivers the beam through the cyclotron shielding wall into a bending magnet system, which selects particles of the proper momentum. Final focusing of the beam is done by a large quadrupole magnet integral with a secondary shielding wall.

Mammalian Cells

A near-diploid transplantable murine lymphoma is maintained at the Lawrence Radiation Laboratory as a peritoneal ascites tumor in female A/Heston mice. Originally derived from the A-2 cell line of Amos (6), the tumor is designated here as L-2. These tumor cells, transplanted to the peritoneal cavities of 16-week-old female LAF_1 mice, were used as the cell system in these experiments. The cells'

stemline chromosome number is 41, with 0.11% polyploidy.⁶ In each case, 10^6 lymphoma cells were injected into the peritoneal cavities of the LAF₁ recipients 3 to 5 days before irradiation.

Irradiation Chambers

HOLDERS for groups of eight mice were constructed by boring eight closely spaced cylindrical holes in a single Lucite block. Each hole was 24 mm in diameter and 75 mm long, closed at each end by Lucite plugs. The wall surrounding each hole was liberally perforated for ventilation. These holders were placed within small Lucite and wood boxes with closely controlled internal temperature, humidity, and light. This control minimized physiological stress which was shown by earlier experiment to interfere with experimental results.

π^- -Beam Irradiation

Groups of four mice each were exposed to the π^- beam at the beam entry position (plateau) and at the Bragg peak position (peak or star region). Energies of π^- in the plateau region were approximately 90 MeV. The π^- beam passed through an ionization chamber (for dosimetric monitoring), and then entered the irradiation chamber. In the irradiation chamber the beam passed first through 3 inches of Lucite (which spread the beam), then through the holder containing four "plateau" mice (mice irradiated in the plateau region of the beam). Beyond this, the beam passed through 4 more inches of Lucite (which further spread the beam and limited the range), then through a second holder of mice which were irradiated in the Bragg peak region ("peak" mice). Finally, the residual beam (now only muons and electrons) passed through a Jordan dosimeter, then left the irradiation chamber. The experimental configuration is shown in both Figs. 1 and 2.

Due to the non-uniform field, whole-body doses ranged from 227 to 265 rads, with an average of 234 rads. In the plateau region, 65% of the dose was attributable to π^- ; the remainder was produced by negative muons and electrons present as beam contaminants. Star region doses, conservatively estimated, ranged from 341 to 360 rads, with an average of 351 rads. Approximately 45% of the star region dose was due to stars resulting from nuclear fission following π^- -nucleus interactions. Approximately 40% was due to π^- not involved in such interactions, and the remainder was due to negative muons and electrons. The dose rate measured in the plateau region only was 5.3 rads/hour.

Lithium fluoride dosimeters, calibrated against both ^{60}Co gamma rays and the plateau pion beam, were placed at various positions around the holder containing the plateau mice. Previous measurements of the plateau and star region doses were made with an ionization chamber to obtain a Bragg peak/plateau ratio. The actual delivered dose in the peak region of the pion beam was calculated from the dose indicated in the plateau region by the LiF dosimeters, and the peak/plateau ratio obtained from the ionization chambers. A discussion of difficulties and errors involved in π^- dosimetry measurements has been presented elsewhere (3, 7).^{3, 4}

Cobalt-60 Gamma-Ray Irradiation

Pairs of lymphoma-bearing mice were exposed to 50, 100, 200, 500, and 1000 R of gamma rays from a 300-curie ^{60}Co source, while they were housed in chambers identical to those used for π^- irradiation. An essentially uniform field was used for whole-body exposure. The dose rates were 5, 12.5, and 20 R/hr.

Controls

Two pairs of mice served as controls for ^{60}Co and π^- irradiations. They were housed in chambers identical to those used for irradiations, and were in every way but radiation subjected to the same stresses as irradiated mice. One pair of mice, subjected to no experimental stress and housed in comfortable and roomy cages in the mouse colony, served as a control for any non-specific effects.

Cytological Examination of Cells

One to three days prior to tumor-induced death of the mice, the mice were sacrificed by cervical dislocation (generally three days after irradiation). The ascites tumor cells were washed from each peritoneal cavity with 1 to 2 cc of physiological saline solution. Cell suspensions were centrifuged at 1000 g for 5 minutes, and the supernatant was removed. To induce osmotic swelling, the cells were resuspended in 0.8% sodium citrate dihydrate at room temperature. After 10 minutes, two volumes of cytological fixative were added; this fixative consisted of 45% propionic acid with 1% lactic acid, in 0.1 N HCl. After 20 to 30 minutes, the cells were centrifuged as above and resuspended in a small volume of 2% lactic-propionic orcein stain. Squash preparations of the stained cell suspensions were then made and examined microscopically at 400 diameters enlargement. Metaphase cells were counted; those thought to be polyploid were examined at 1500 diameters and were counted separately. The number of polyploid cells seen during examination of 4000 metaphase cells per mouse was recorded.

Data Reduction

All calculations were performed on a programmable desk computer (Olivetti-Underwood Programma 101). Several kinds of statistical programs were utilized, employing "t" distributions and "small-number" methods when necessary (8, 9).

Within a dose-rate and dose group, differences in polyploid frequency were assessed by t tests for significance between members of a pair of mice. When differences were not significant at $\alpha = 0.05$, the data were combined. Combined data for dose-rate groups were assessed for significance of intergroup differences. When possible, dose-rate data were combined so that we could derive mean polyploid frequencies as a function of dose alone, based on large numbers of cells. Similarly assessed and combined control values, amounting to 0.11% polyploidy, were subtracted from the derived experimental values. Ninety-five percent confidence intervals were calculated for the corrected experimental values derived from π^- and ^{60}Co irradiations. For the ^{60}Co data, the equation expressing the percent polyploidy regression on dose was calculated. The 95% confidence-of-prediction intervals around the ^{60}Co regression line were calculated.

After plotting both ^{60}Co and pion data on linear graph paper, we drew lines from the origin to the π^- data points. We then compared the slopes of these lines with the slope of the ^{60}Co regression line to obtain initial estimates of π^- -beam RBE.

RESULTS

The data relating mouse number, dose, dose rate, polyploid cell frequencies, total cells observed, and t values for differences, are given in Table I. The reduced data, displaying ^{60}Co data points, the ^{60}Co regression, the standard-error-of-estimate lines, and the 95% confidence interval for all points, along with the two π^- -beam data points, are shown in Fig. 3.

On the assumption that muon and electron contaminants had an RBE of 1 (relative to ^{60}Co gamma rays), their contribution was subtracted from the induced polyploidy; this left an estimate of effects due to pions alone. With an RBE of 1 assumed for π^- mesons not undergoing π^- -nuclear interactions, the contribution of such pions to induced polyploidy was subtracted; this provided an estimate of star effects. Lines were drawn from the origin to these two points obtained by subtraction; the comparison of their slopes with the slope of the ^{60}Co regression line yielded RBE estimates both for all pions and for stars alone. The derived π^- -beam data points, with associated slopes of lines, are shown with the ^{60}Co regression line in Fig. 4. The geometric method for deriving the 90% confidence interval for π^- -beam RBE is shown in Fig. 5.

It can be seen in Fig. 3 that the π^- -plateau value does not differ significantly from the ^{60}Co regression line value. The RBE of plateau region relative to that of ^{60}Co gamma rays, is therefore estimated to be 1.

The π^- data point in the star or peak region is far higher than both the π^- plateau region value and the value predicted on the basis of the ^{60}Co regression equation. The peak-plateau difference is significant at $\alpha \leq 10^{-5}$. In Fig. 4, the slope of the peak π^- line is 2.15 times that of the ^{60}Co

regression line. Thus, peak-region π^- beams with approximately 15% of delivered dose due to muon and electron contaminants have an RBE estimated as 2.15.

In this linear model, the ratio of dose predicted (by the ^{60}Co regression line, given the observed polyploidy) to the actual dose is geometrically equivalent to the ratio of the appropriate slopes. With the extreme 95% confidence limits used for both the π^- peak data point and the ^{60}Co regression line, two doses were predicted from the observed peak π^- data. These doses, $D'_p = 568$ rads and $D''_p = 1008$ rads, and the 95% confidence limits used in their derivation, are shown graphically in Fig. 5. The dose ratios $D'_p/D = 1.6$ and $D''_p/D = 3.1$ represent the estimated range of π^- -beam RBE values to be expected 90% of the time in replicate experiments.

From Fig. 4, it is seen that slope comparisons between derived π^- data and the ^{60}Co data permit derivation of $\text{RBE} = 2.37$ for pion effects only, and $\text{RBE} = 3.64$ for star effects only. Expected ranges for these values were not computed, as assumptions described below introduce an unknown degree of inaccuracy.

DISCUSSION

The preliminary experiments of Loughman et al.⁵ indicated that the peak frequency of radiation-induced polyploidy in the cell system used occurs on the third to fourth post-irradiation day. This is in agreement with the observations made by Yu and Sinclair (10) using x-irradiation of hamster cells in vitro. We therefore think that the polyploid frequencies used in this report are maxima.

The data of Yu and Sinclair (10) are also consistent with linearity of the dose-response relationship in the dose range used in these experiments. However, these authors indicate that the response is non-linear over-all, rising at higher doses than used in our work. Extrapolation of our linear dose-response curve outside the dose range used should therefore be done with caution.

The determination of RBE presented here is subject to error arising partly from the statistical treatment and partly from the use of certain assumptions. For estimation of the range of π^- -beam RBE values, the predicted dose values were derived from observed effect, using the line of regression of effect on dose. Normally this estimate should be derived from the alternate regression of dose on effect. The approach used here is justified by the closeness of the correlation coefficient to unity ($r = 0.998$). The two regression lines differently derived are thus essentially identical.

The RBE estimates presented in this work are ratios of slopes except for the 90% confidence interval for total peak region beam RBE, which is derived from an equivalent geometric method. This approach was necessitated by the fact that confidence limits around regression lines are hyperbolic. That is, the slope of the confidence-limit line

is variable, and may deviate significantly from the slope of the regression line at dose values distant from the mean dose.

Accuracy in measurement of the fraction of peak-region dose due to various components of the pion beam is assumed. If muon and electron dose contributions were underestimated in these measurements, the RBE values for pions only and for stars only would be overestimated.

In subtracting the effects of muon and electron contaminants to arrive at an RBE for the pure π^- beam, we assumed that the RBE of muons and electrons equalled 1 relative to that of cobalt-60 gamma rays. Since others have shown that the RBE of negative muons is approximately 0.8 (4, 5), and cobalt-60 gamma rays have an RBE of 0.8 (11, 12), our assumption should not introduce any significant inaccuracy.

The RBE of π^- 's slowing very near the end of their range, but not stopping in the test volume (i. e., position of the peak mice), is not known. It is probably higher than 1 relative to that of cobalt-60 gamma rays. When an RBE of 1 is assumed for these particles, the RBE for stars only is overestimated.

It may not be possible to obtain a pure π^- beam for radiotherapy purposes, because of the decay of pions into muons. Nonetheless, a Bragg-peak region relatively very rich in star events may be obtained by utilizing a π^- -momentum spectrum narrower than that used in these experiments. If this is done, the width of the Bragg-peak irradiated volume will be reduced to less than the 2 inches utilized in this work, and the π^- LET will be raised, and a concomitant increase in RBE is to be expected. However, the calculation of RBE presented here for stars only indicates that an RBE of 3.64 may be an upper limit to this increase relative to polyploidy induction.

Micke et al. (4) give an RBE of 3.23 for their π^- beam, in disagreement with the values given here. Their π^- beam was of such high energy that a significant neutron flux from the cyclotron could have been present. This undetected radiation may have influenced their results and given a plateau RBE even higher than our peak region RBE. Aside from this speculation, at least two factors may account for the discrepancy.

The biological material used by Micke et al. --dormant seeds-- would accumulate radiation damage without significant repair until germination. Our material was a dynamic cell population in which radiation damage to any one cell was distributed between its daughter cells appearing during the lengthy radiation episode. It may be safely presumed that repair processes were in operation as well; such aspects of a dynamic cell population would conspire to reduce the observable damage effects, lowering RBE estimates from the values which might be obtained in the absence of division and repair.

Micke et al. also report no reduction in germination frequency in their irradiated seeds, implying little or no cell death. In unpublished experiments done on the same cell populations utilized in our experiments, Feola et al.⁸ have found a significant amount of post-irradiation cell death, increasing exponentially with radiation dose. In addition, others such as Fowler (2), Brustad (13), and Todd (14) have given plots of RBE versus LET for various cell survival frequencies. These indicate that at the low LET values of plateau-region π^- beams, RBE does not depend heavily upon cell survival. However, at the high LET values associated with star events in the Bragg-peak region of a π^- beam, decreased survival produces a reduced RBE value. These facts may explain why our

value of RBE for a high LET Bragg-peak irradiation is lower than that found by Micke et al. for a high-energy beam with a low LET.

The passage of a single high LET particle through a cell nucleus is sufficient to kill the cell (14). Therefore a large fraction of the induced polyploidy in the pion beam's Bragg-peak region must be a secondary event, probably produced by low LET components of the star event, by pions with low LET slowing near the end of their range, or by high LET particles interacting only with the cells' cytoplasm. The polyploid cells produced in these experiments eventually disappear from the cell population,⁸ and polyploidy is thus probably eventually lethal in terms of the cells' proliferative capacity. Bragg peak RBE from survival curves should thus measure direct effects of high LET pions in producing cell lethality, plus the effect of lethality due to polyploidy (and other indirectly lethal phenomena). The production of cell-lethality from all causes by Bragg peak negative pions should therefore be relatively greater than from the production of polyploidy alone. The RBE of Bragg peak negative pions, based on survival curves, should thus be higher than the RBE reported here. Support for this contention is found in the experiments of Feola et al.,⁸ who used the same irradiated cell populations described in this report. They found a Bragg peak region RBE for negative pions of 5.4, based on survival curves.

Various factors in this work cause an overestimation of the RBE values for the Bragg peak of a π^- beam in terms of radiation-induced polyploidy; these RBE values in turn may well cause an underestimation of the RBE which would be obtained from survival curves. The latter effects would be very important in tumor radiotherapy.

CONCLUSIONS

The RBE of the plateau region of the contaminated π^- beam relative to cobalt-60 gamma rays was estimated to be 1. The RBE of the Bragg peak, or star region, of this beam was estimated to be 2.15, with a 90% confidence interval of 1.6 to 3.1.

The estimated RBE of π^- 's only, relative to cobalt-60 gamma rays, was 2.37. The RBE for stars only was 3.64, and represents an estimated upper limit to the RBE for polyploidy induction of π^- 's in the Bragg peak region.

From theoretical considerations, supported by preliminary observation, it seems likely that the RBE of π^- 's for all cell lethal effects may be higher than those based on polyploidy induction. The high RBE of star region π^- beams, with the reduced O_2 effect which should be realized from this region's high LET, suggest that π^- beams may be much more effective in radiotherapy than other available types of radiation.

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Table I.
Mouse Tumor Polyploidy--Experimental Data

Type of radiation ^a	Dose ^b	Dose rate ^c	Mouse No.	No. of polyploid cells ^d	No. of cells counted ^e	t for the Difference between: ^f Mice Dose-rate		Percent polyploidy ^g (combined data minus control)	95% confidence interval for percent polyploidy
⁶⁰ Co gamma	50	5	1	8	4000	0.84	---	0.05	0.02-0.13
			2	5	4000				
	100	5	3	10	4000	0.25	---	0.16	0.10-0.25
			4	9	4000				
			12.5	5	13				
	200	5	6	died	---	0.93	0.92	0.27	0.20-0.36
			7	13	4000				
			8	18	4000				
			12.5	9	16				
	500	12.5	10	13	4000	0.56	---	0.83	0.65-1.05
			11	41	4000				
			12	34	4000				
	1000	20	13	57	4000	1.50	---	1.53	1.23-1.82
			14	74	4000				
π ⁻	234 (plateau)	5.3	15	21	4000	(all pairs)	---	0.37	0.27-0.49
			16	18	4000				
			17	17	4000				
	351 (peak)	?	18	died	---	1.82	---	1.29 ⁱ	1.06-1.56
			20	46	4000				
None	Absolute control		21	died	---				
			22	died	---				
	⁶⁰ Co control		23	3	4000	(all pairs)	---		0.08-0.15
			24	died	---				
			25	7	8000				
Pion control		26	8	8000	< 1.1	---		(X̄ = 0.11)	
		27	11	8000					
Prior controls			28	8	8000				
			26	23 439					

^a Cobalt-60 is 300 Ci at various distances; π⁻ mesons were cyclotron produced, and contaminated with μ⁻ mesons and relativistic electrons.
^b Cobalt-60 in roentgens; π⁻ mesons in rads. These units were experimentally determined to be essentially equivalent.
^c Cobalt-60 in R/hr; π⁻ mesons in rads/hr.
^d Before correction for control values. Deaths resulted from tumor growth, not radiation.
^e Mitotic cells in which degree of ploidy could be estimated.
^f Here t is (difference between proportions)/(est. std. error of difference between proportions). Differences are not significant for t < 1.96, with α = 0.05.
^g From each value in Column 5, 4.4 cells were subtracted for each 4000 cells in Column 6. [The 4.4 cells is the mean control value (0.11%) multiplied by 4000 cells.] The corrected Column 5 values are then summed for each dose, and expressed in Column 9 as a percent of all cells observed for that dose.
^h Corrected as for g; limits obtained for α = 0.05 by method of approximation to the binomial expansion.
ⁱ Here t for difference between peak and plateau values of polyploid frequency (corrected for control values) = 7.52 and α < 10⁻⁵. The difference is therefore highly significant.

FIGURE LEGENDS

- Fig. 1. Schematic drawing of arrangement of cyclotron, pion beam, magnets, experimental chambers, and dosimeters. Explanation in text.
- Fig. 2. Close-up view of mice in holders with dosimeters and absorbers. Environmental control box not shown. Pion beam enters from the right. Holder 2 is in Bragg peak position; holder 3 is shown closer to the beam area than it actually is for control mice.
- Fig. 3. Polyploidy induction in lymphoma ascites cells as a function of dose: Comparison of data from Co-60 gamma irradiation with data from π^- -beam irradiation. O, cobalt-60 data. \bullet , \square , π^- data. Vertical bars are 95% confidence intervals. Heavy line is drawn from the equation expressing the regression of polyploidy on dose: (% polyploidy) = [0.00155 (dose) - 0.0081]. Dashed line is regression line \pm 1 standard error of the estimate ($S_{y \cdot x} = 0.03$). Correlation coefficient = 0.998. Rad dose in plateau is 234; estimated rad dose in peak is 351.
- Fig. 4. RBE of fractions of π^- beam. Solid line is regression line from ^{60}Co data. Solid squares are values of polyploidy vs dose for whole peak region π^- beam, pions only in peak region, and "stars" only in peak region. Respective RBE values are ratios of slopes of dashed lines to the solid (^{60}Co) line. Slopes of relevant lines are represented by b_1 , b_2 , b_3 , and b_4 .
- Fig. 5. Estimate of 90% confidence interval for RBE peak region of beams. D, actual dose in peak region of π^- beam = 351 rads (estimated). D_p , hypothetical dose which would have produced the π^- beam peak region polyploidy, predicted by ^{60}Co regression

equation. D'_p and D''_p , dose as for D_p above, predicted on basis of upper and lower 95% confidence interval for prediction (^{60}Co data), and the 95% confidence interval for the π^- beam polyploidy.

Dashed lines, 95% confidence interval for prediction of polyploidy from observed dose (^{60}Co data). These lines are derived from $(Y = bX + a) \pm e$, where $(Y = bX + a)$ is the regression equation in Fig. 3, and e is a hyperbolic function incorporating the random distributional error of Y .

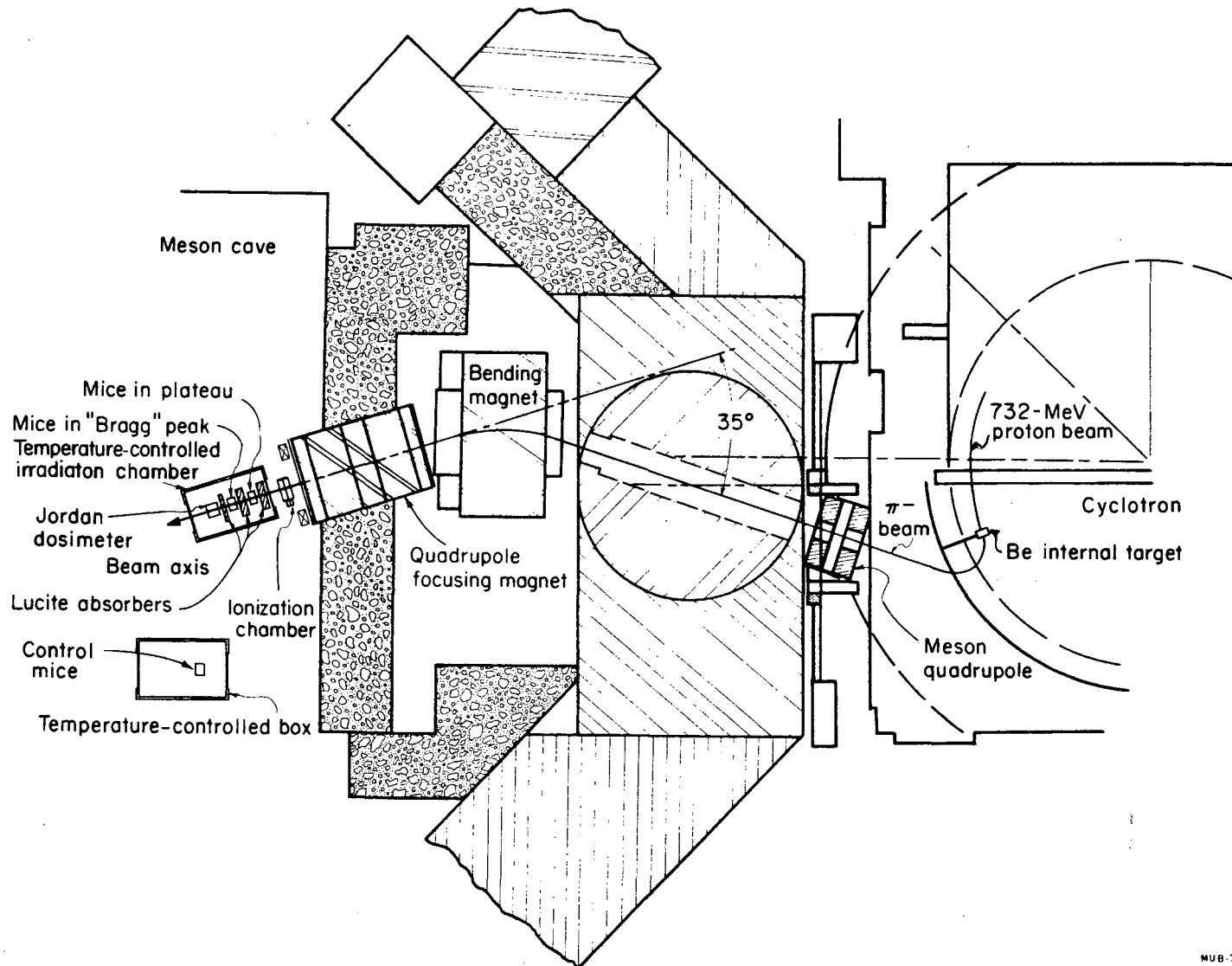
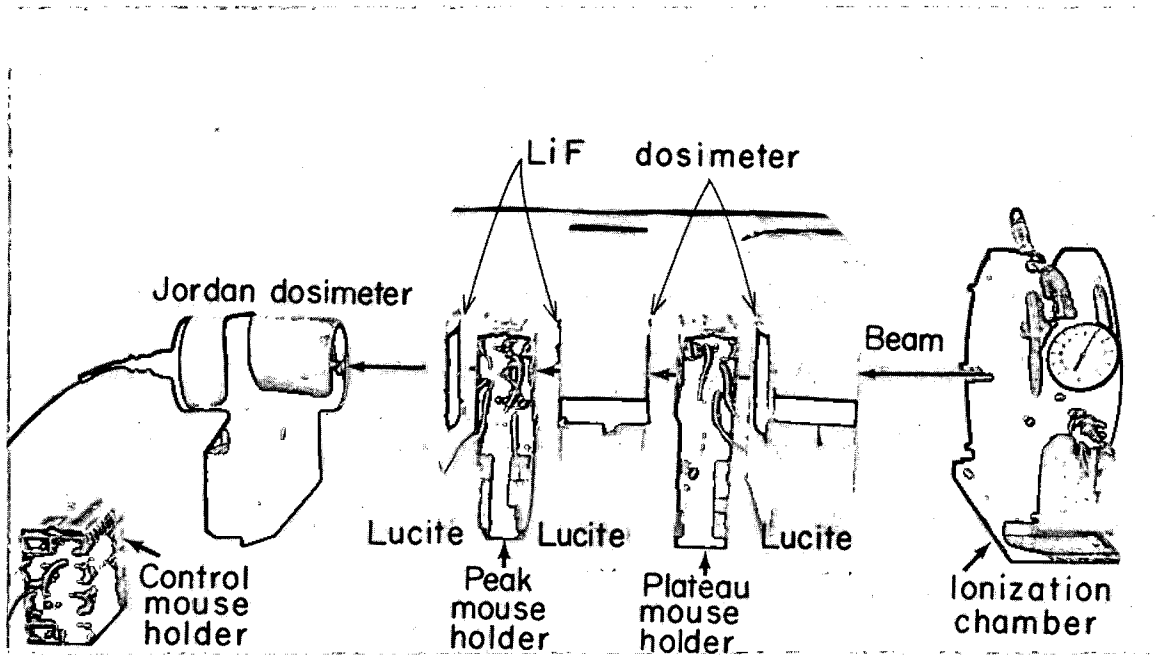
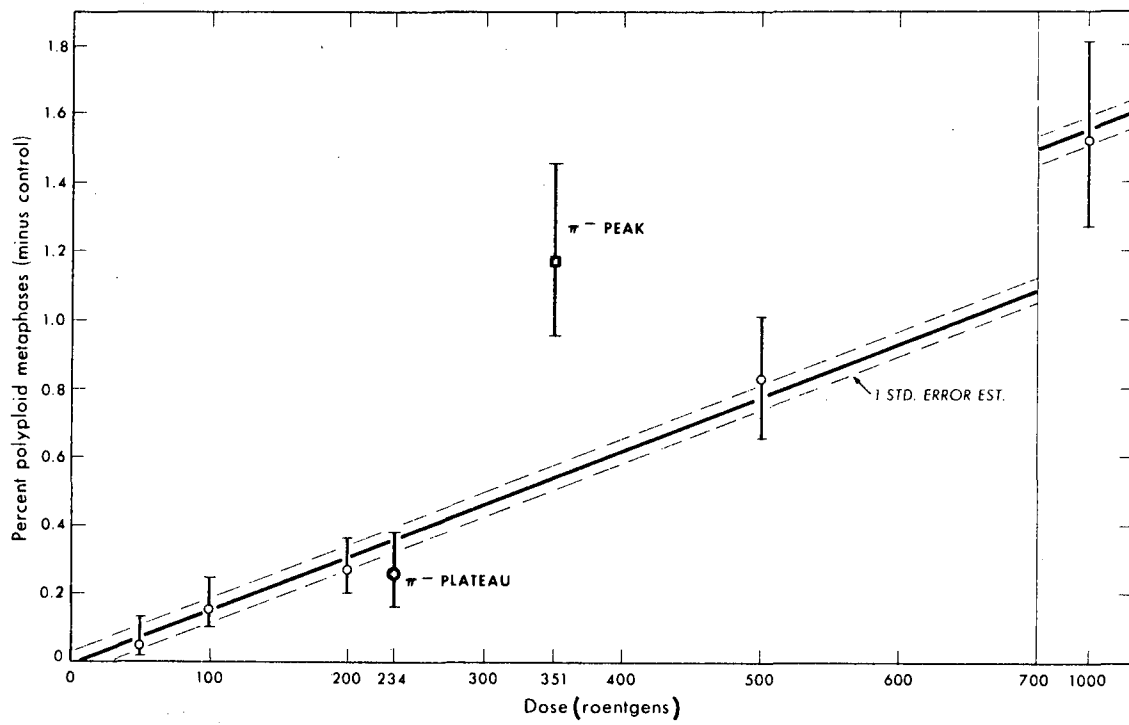


Fig. 1



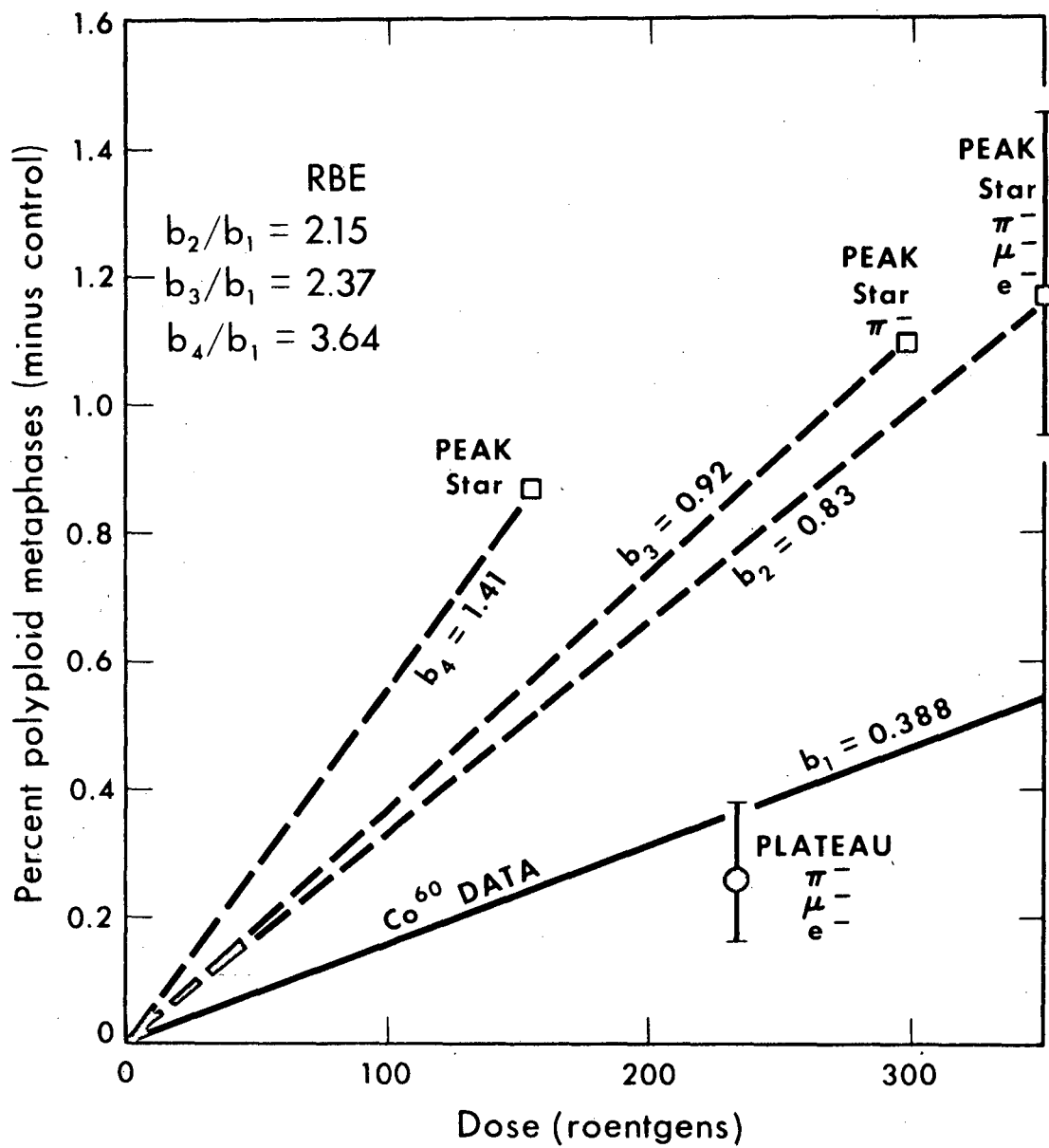
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Fig. 2



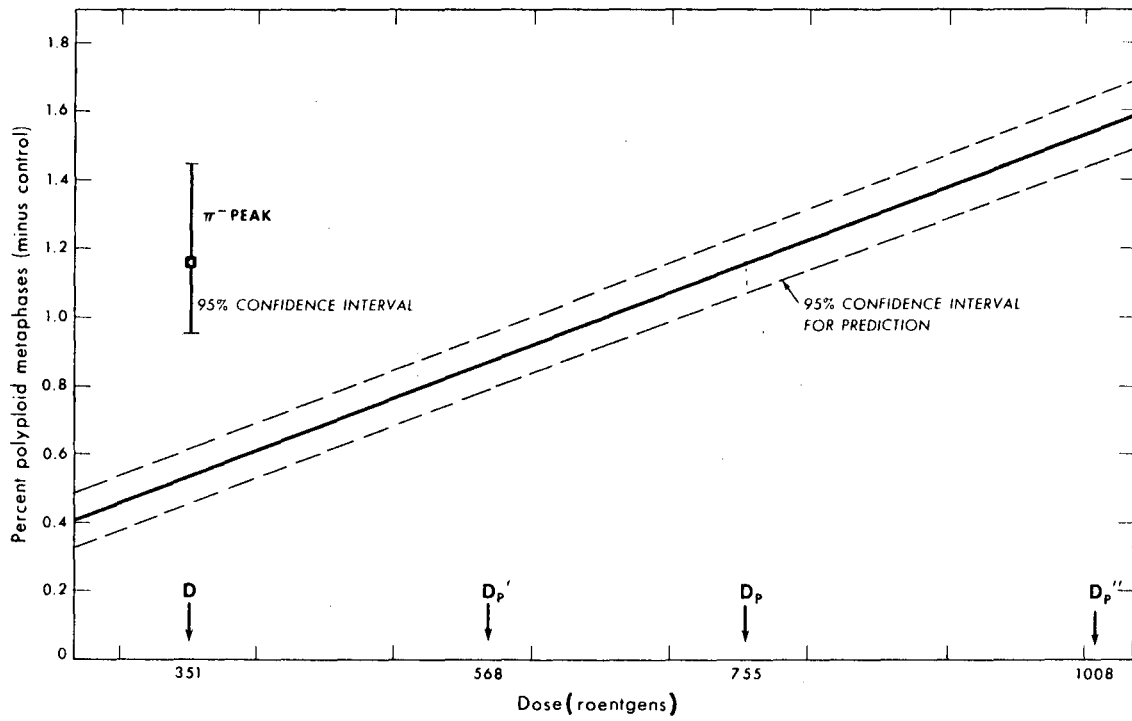
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Fig. 3



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Fig. 4



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Fig. 5

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