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GROWTH KINETICS OF Bp8 MOUSE ASCITES SARCOMA AFTER SINGLE DOSES OF WHOLE BODY IRRADIATION

II. Analysis of the progression of cells through the cell cycle

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The proliferation kinetics of the undisturbed Bp8 mouse ascites sarcoma growing in vivo have been described by CAO et coll. (1982 c).

In part I on the cell kinetics of the irradiated ascites tumour both the changes in the total number of malignant and normal cells and the changes in the proportion of cells in the various parts of the cell cycle following doses of 1.75 to 8 Gy have been described (CAO et coll. 1982 a). Based on these data, a detailed analysis of the cell kinetics was performed and is now presented.

Experimental procedures

Details of the experimental conditions have been described previously (CAO et coll. 1982 a, c). Briefly, male NMRI mice were inoculated intraperitoneally with 18×10^6 Bp8 ascites sarcoma cells. On the fourth day after inoculation the animals were exposed to whole body irradiation with 1.75, 2.5, 5.0 and 8.0 Gy (250 kV, 15 mA, SSD 50 cm, 0.5 mm Cu added filtration, 1.3 Gy/min). At various times after irradiation the total number of cells was estimated from the measured ascites volume and the cell concentration. The number of cells in the various parts of the cell cycle was estimated from the total number of cells and the proportion of cells in the cell cycle as measured by flow-cytofluorometric DNA analysis and the mitotic index.

The calculation of the cell flow through the cell cycle is based, as described previously (CAO et coll. 1982 c), on the knowledge of the changes in the total number of cells in the various compartments of the cell cycle with time. These data are obtained from the total number of cells measured and the proportion of cells in the various parts of the cell cycle at different times. Let us, as an example, consider the changes in the unirradiated tumour between days 4 and 5 after inoculation of tumour cells. The total number of cells on day 4 is found to be 482×10^6, while this total on day 5 increased to 820×10^6. The proportion of cells in G_1, S-phase, G_2 and M are on day 4: 28.4, 58.8, 10.6 and 2.2 per cent and on day 5: 31.0, 55.5, 11.6 and 2.0 per cent. Thus the total number of cells in G_1, S-phase, G_2 and M is on day 4: 136.9×10^6, 283.4×10^6, 51.5×10^6 and 10.6×10^6 and on day 5: 254×10^6, 455.1×10^6, 95.1×10^6 and 16.4×10^6. Since the total number of cells increased by 338×10^6 cells, this number of cells was produced by mitosis and twice this number, 676×10^6, entered G_1. Since 254×10^6 G_1 cells were found on day 5, 559×10^6 cells had left G_1 and progressed to the S-phase. The flow from S-phase into G_2 and from G_2 into M is calculated in a corresponding way. In this way the flow of the total number of cells from one compartment to the following one during a specific time period can be calculated. The average of the

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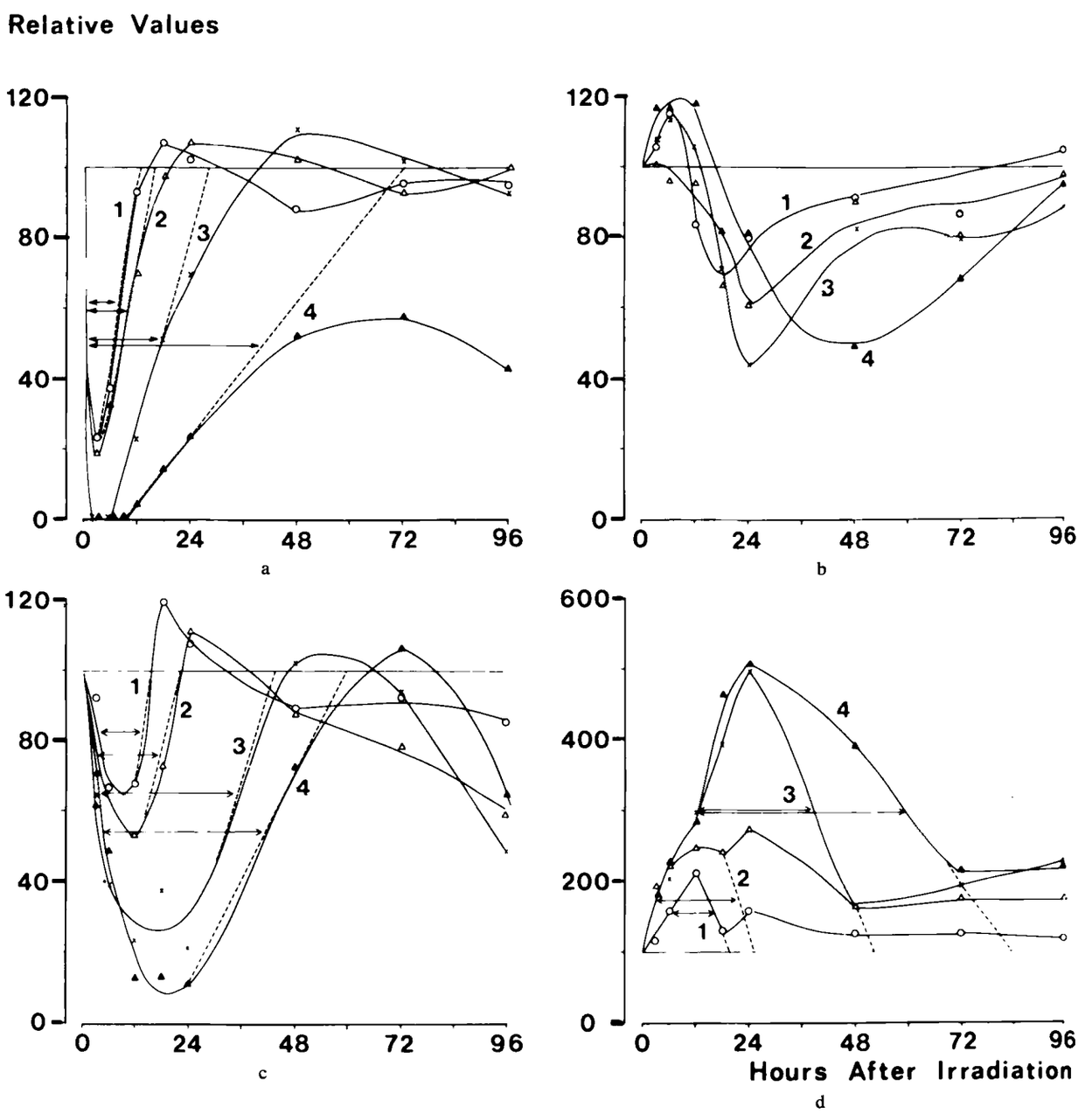

Fig. 1. Total numbers of cells in the various compartments of the cell cycle of the Bp8 mouse ascites sarcoma after single doses whole body irradiation with 1.75 (1=○), 2.5 (2=△), 5 (3=×), and 8 (4=▲) Gy. a) Mitosis, b) G_1, c) S-phase, d) G_2. The values are normalized and expressed as percentages of controls. Mean values of 4 to 8 mice.

inflow and outflow per unit time expresses the mean flow rate through a compartment. This value divided by the number of cells in this compartment, i.e. the pool size, is called the relative flow rate. The inverse of the relative flow rate is equal to the mean duration the cells spend in the compatment. In the example a duration of 7.8 hours was calculated for G_1, while the duration of the S-phase was 18.7 hours, of G_2 4.8 hours and mitosis 1.0 hours. These estimations are valid when the growth fraction is 1.0 and no cell loss is present.

Results

Mainly two events are responsible for the rapid initial disturbance of the cell flow through the cell

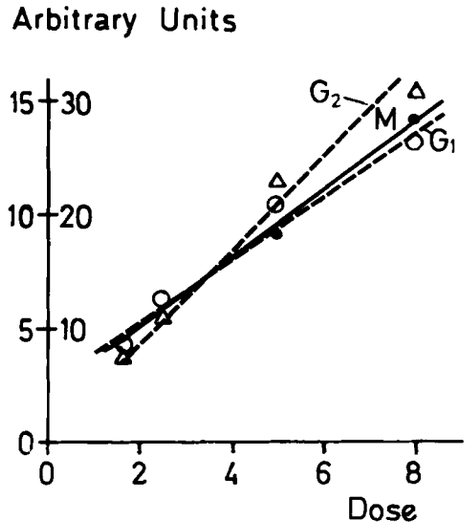

Fig. 2. Relationship between irradiation doses in Gy and the mitotic delay (●), the G_1 depletion (○) and the G_2 blockage (△) expressed as cell number-time areas. On the ordinate, the square root of the areas is given (arbitrary units). The values to the left on the ordinate are valid for M and G_1, the values to the right for G_2.

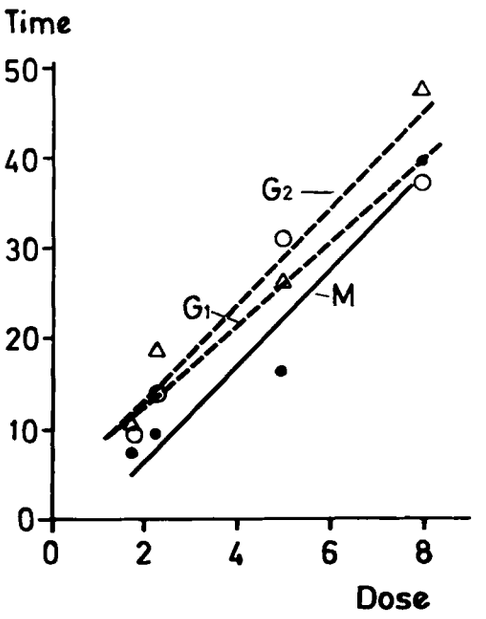

Fig. 3. Relationship between irradiation doses in Gy and the mitotic delay time (●), depletion time (○) and duration of the G_2 blockage (△) in hours. The values were obtained from the half-values between the mid-points of the descending and ascending curves as indicated by arrows in Fig. 1.

cycle following irradiation: the G_2 blockage and the mitotic division delay, which in turn results in the depletion of the G_1 compartment. The following factors were analysed: (1) The changes in the number of cells in the various compartments of the cell cycle, (2) the outflow rate of the cells from one compartment to the following one, (3) the outflow rate related to the pool size and (4) the mean duration of the various parts of the cell cycle. All these values were normalized and expressed as a percentage of the values for the controls. Since the number of cells in the controls reached the plateau level on day 7 and since consequently flow-rate calculation is possible only up to this time, calculation of the irradiated cell populations are also restricted to this period, i.e. the 3 days after irradiation.

The changes in the distribution of cells in the various compartments following irradiation with doses of 1.75, 2.5, 5 and 8 Gy appear in Fig. 1. About 30 min after irradiation the mitotic index (Fig. 1a) decreased significantly and reached minimum values at about 3 hours. While after 1.75 and 2.5 Gy the mitotic index 6 h after irradiation had already increased, a complete block for up to 6 and 9 hours was found after 5 and 8 Gy. The reappearance of the mitotic cells was characterized by an overshoot up to doses of 5 Gy and the fact that after 8 Gy the mitotic index did not reach the original level. These

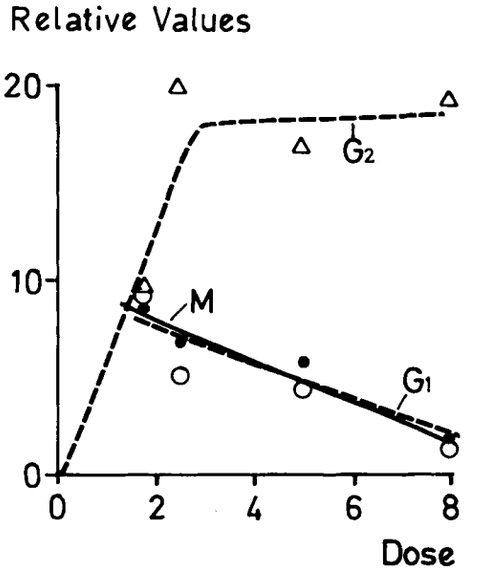

Fig. 4. Relationship between irradiation doses in Gy and the accumulation rate of the cells in M (●), G_1 (○) and G_2 (△). The data are given as relative values.

two factors complicate the quantitative evaluation of the mitotic division delay. The rate of increase of the mitotic index during the first few hours is, however, approximately linear. These slopes are indi-

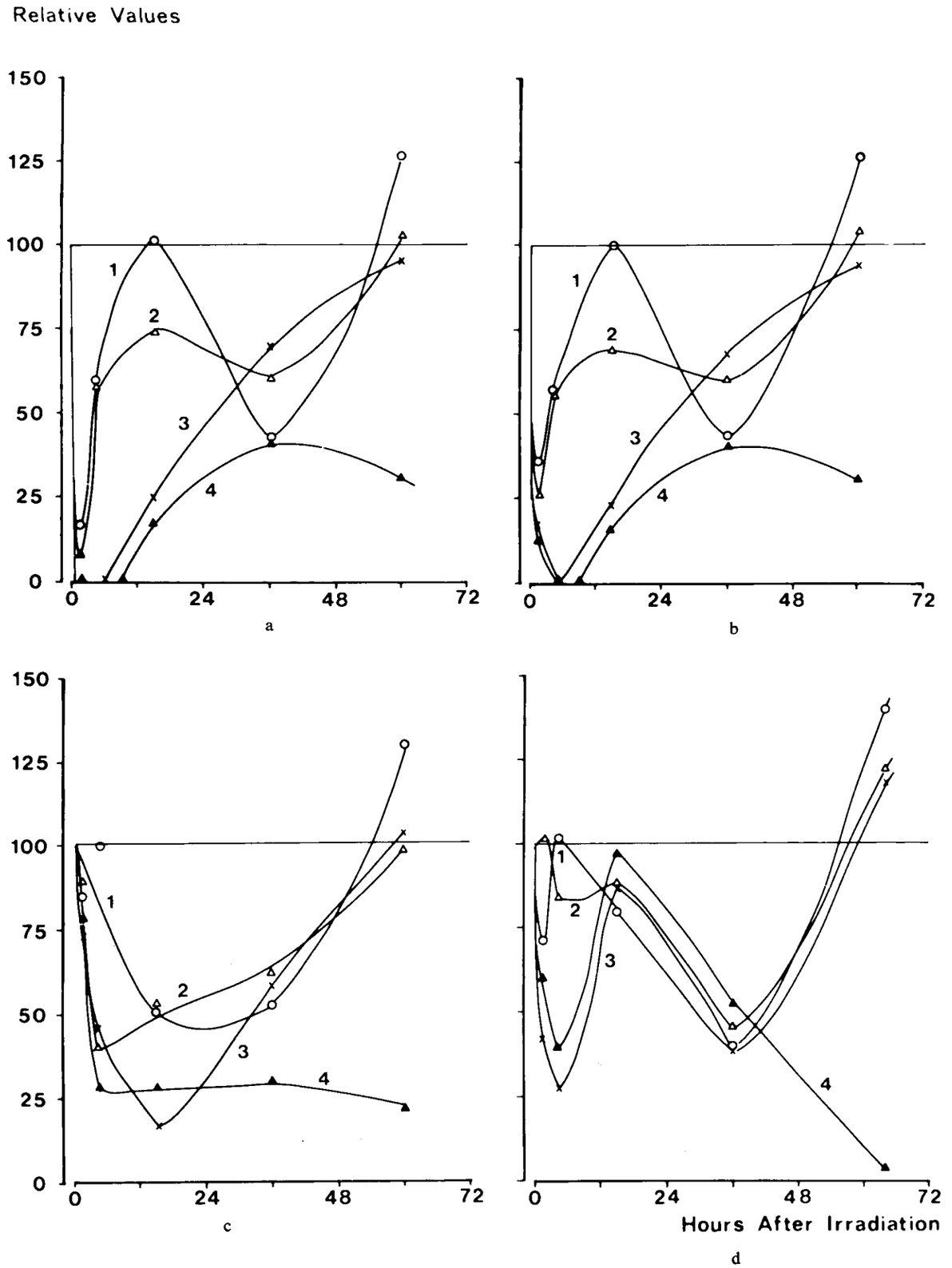

Fig. 5. Flow rate of the total number of cells from one compartment of the cell cycle to the following one after irradiation doses of 1.75 (1=○), 2.5 (2=△), 5 (3=×) and 8 (4=▲) Gy. The values are normalized and expressed as percentages of controls. a) $G_2 \rightarrow M$, b) $M \rightarrow G_1$, c) $G_1 \rightarrow$ S-phase, d) S-phase $\rightarrow G_2$.

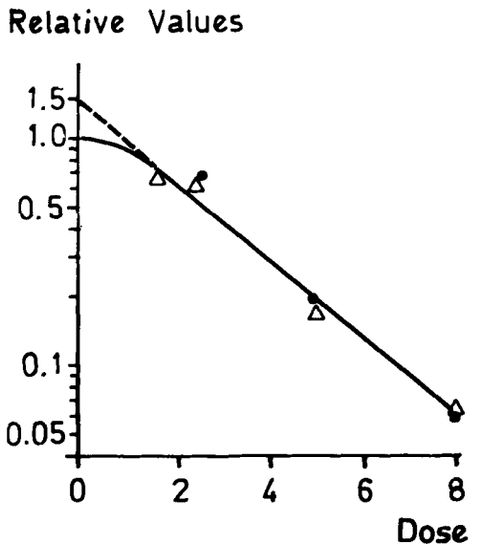

Fig. 6. Dose response curve of the outflow rate of the cells from G_2 (△) and M (●) after irradiation doses of 1.75 to 8 Gy. The values are given as a fraction of the control values (=1.0).

cated in Fig. 1a. When extrapolated to the control level, together with the curve of decreased of the mitotic index, areas were obtained from which the total mitotic delay can be quantified. In a corresponding way the depletion of the G_1 compartment has been quantified (Fig. 1b). The accumulation of cells in the G_2 compartment was calculated from the area given by the curve of the increase in the cell number and the curve of the decrease extrapolated to the control level (Fig. 1d).

The areas for the mitotic delay and the G_1 depletion were about the same, while the accumulation area for G_2 cells was about 5 times greater than these areas. Dose response curves of the areas follow a quadratic function as is shown by the linear relationship between the square root of the areas and the doses (Fig. 2). The delay times of cell progress calculated for the 50 per cent level of the decrease in the mitotic and G_1 cells and the 50 per cent increase in the G_2 cells, as indicated by arrows in Fig. 1, are linearly related to the dose (Fig. 3). The delay times per Gy were dose independent and had a mean value of 4.0 h for the mitotic cells, 5.5 h for the G_1 cells and 6.1 h for the G_2 cells. It should be noted that following 8 Gy the calculated areas for the mitotic division delay and the delay time are based on the extrapolated value of the slope. In fact, the mitotic index never reached the control level. In contrast, the G_1 values reached the control level 72 h after irradiation. This discrepancy can be explained by a decrease in the mitotic duration to about half the normal or an increase in the duration of G_1 to double the normal value. For the doses below 8 Gy no such differences were found.

The decrease in the flow rate of the cells in G_1 during the time of the complete block of the mitotic cells was calculated to be about 10 per cent per hour and seems to be independent of the dose. Thus the mean duration for G_1 after irradiation was about 10 h which can be compared with the duration of about 8 h for the unirradiated cell population.

The slopes of the accumulation rate of the cells in M and in G_1 were about the same and show a dose dependent decrease (Fig. 4). The slope of the accumulation rate of the cells in G_2 deviated markedly with increasing values after 1.75 Gy and reached a maximum value after about 2.5 Gy (Fig. 4). The accumulation rate per Gy for the cells in M and G_1 showed non-linear decreasing values from about 5 per cent per hour for 1.75 Gy to 0.2 per cent per hour for 8 Gy. The accumulation rate per Gy for G_2 cells increased from 5 to 8 per cent per hour following 1.75 and 2.5 Gy and decreased to 3.3 and 2.4 per cent per hour following 5 and 8 Gy.

The proportion of cells in S-phase (Fig. 1c) increased during the first 12 hours, reflecting the partial inhibition of the DNA synthesis (data not given). It decreased thereafter in a dose dependent way as a consequence of the G_1 depletion.

The outflow rate of cells from one compartment to the following one is shown in Fig. 5. These values were calculated in different sets of experiments for the time periods 0 to 3, 3 to 6, 6 to 24 in the first set and 12 to 18 h after irradiation in the second set. The values from the last two periods were generally in good mutual agreement and are given as average values. In addition, values from the periods both of 24 to 48 and 48 to 72 h were calculated.

The outflow rate of cells from G_2 (Fig. 5a) and M (Fig. 5b) are nearly identical and are characterized by a dose dependent decrease in the flow rate after release of the block. After 1.75 and 2.5 Gy the outflow rate was biphasic with a minimum between 24 and 48 h while after 5 and 8 Gy the outflow rate increased to maximum values after about 48 to 72 hours. The outflow rate of the G_2 and M cells after release of the blockage, calculated for the 3 to 6 h intervals for 1.75 and 2.5 Gy and for the 6 to 24 h periods for 5 and 8 Gy, shows an exponential dose response relationship. This curve has a shoulder

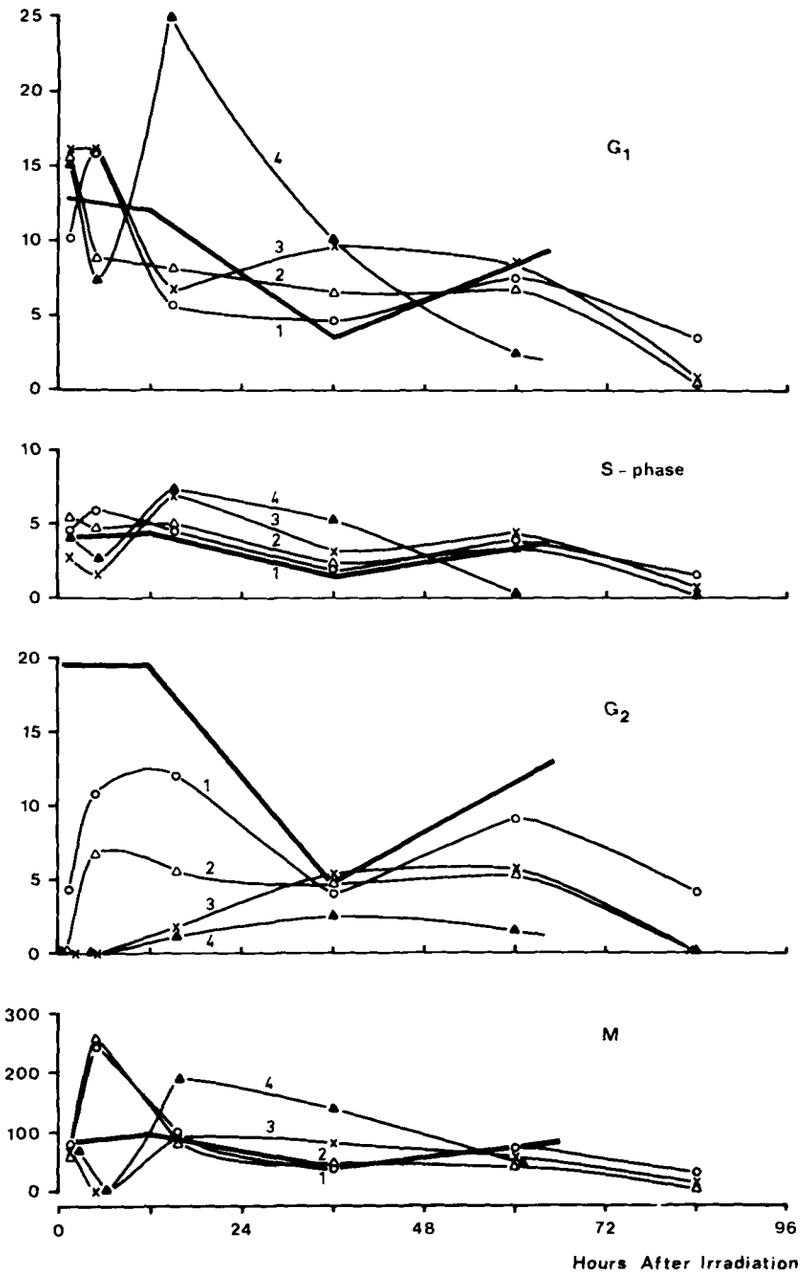

Fig. 7. Outflow of cells per hour from G_1, S-phase, G_2 and M as a percentage of the pool size after irradiation doses of 1.75 (1=○), 2.5 (2=△), 5 (3=×) and 8 (4=▲) Gy. Values for unirradiated cells are indicated by solid lines.

with an approximate D_0 of about 2.5 Gy, a Dq value of 1 Gy, and an extrapolation number of 1.5 (Fig. 6).

The outflow rate of cells from G_1 to S (Fig. 5c) reached minimum values after some hours due to the partial or complete blocking of the inflow of cells, and increased again following the inflow of cells after the release of the mitotic division delay. The outflow rate was nearly dose independent following doses up to 5 Gy. It reached normal values after 48 to 72 h although it remained at a 25 per cent level following 8 Gy (Fig. 5c). The outflow rate from S to G_2 (Fig. 5d) was, after the initial decrease, nearly normal in the time interval of 6 to 24 h after irradiation. It decreased again between 24 and 48 h, now reflecting the mitotic division delay about 24 h earlier. Control levels were reached between 48

and 72 h following 1.75 to 5 Gy. Eight Gy results in a further decrease, now caused by the partial persistance of the G_2 blockade.

In relating the outflow rate to the pool size (Fig. 7), the degree of dependence of the flow rate on the number of cells available was analysed. For the relative flow rate from G_1 and S-phase no major differences from normal conditions were found except for the flow from G_1 12 to 18 h subsequent to irradiation with 8 Gy, during which time the pool size decreased to a minimum value of about 5 per cent. The relative flow rate from G_2 decreased in a clearly dose-dependent way at least up to 24 hours. Whether the decrease in the relative flow rate of the unirradiated cells 5 to 6 days after transplantation at 36 h is accidental or not is an open question. The relative outflow rate from M, which, due to the small pool size, is normally the highest one, doubles between 3 and 6 h following 1.75 and 2.5 Gy while following 5 and 8 Gy no mitotic cells were present.

By substracting the relative outflow rate from the relative inflow rate the dynamics of the net changes in the pool size were established and appear in Fig. 8. In unirradiated cells, represented by solid lines, the pool size has an hourly increase of about 2 per cent. By comparing the values with those in Fig. 5, it is obvious that after irradiation the early negative values in G_1 are caused by the low inflow rate followed by an increase in the inflow rate. The generally negative values in the S-phase at 15 h are a consequence of a relatively low inflow rate. The high values up to 24 h in G_2 are mostly due to the decrease in outflow rate. After negative values in M the positive balance is generally due to a high relative inflow rate.

The mean durations of the various parts of the cell cycle and the total cell cycle times are shown in Fig. 9. As a rule the durations increased up to about 6 h after irradiation in a dose dependent way. Due to the complete stoppage of the flow in G_2 and M up to 6 and 9 h following irradiation the durations of G_2 and M and the total cell cycle time were infinitely prolonged. The further changes had an oscillating behaviour around the control level except G_2, in which, following 1.75 to 5 Gy the control level is reached first after 48 to 72 h while after 8 Gy the duration again increased at this time. The total cell cycle time was prolonged by approximately 50 per cent up to about 36 hours. Normal or decreased cell cycle times appeared between 48 and 72 h except following 8 Gy, where the duration again increased. The main contribution to the increase in the duration 12 to 36 h after irradiation was given by the prolongation of the G_2 phase.

Discussion

Early changes in the flow of cells through the cell cycle following irradiation have been analysed in a large number of investigations. A comprehensive summary is given by OKADA (1970). Generally, the following changes were found for the first post-irradiation cell cycle: No effects in the progression from M to G_1, no or slight depression in the progression from G_1 to S, a prolongation of the S-phase and blockage in G_2. As a consequence, the mean duration of the cell cycle increases. However, considerable differences exist between different experimental systems.

The observations on the progress of cells from G_1 to S and S to G_2 have generally been based on continuous or pulse labelling techniques with radioactive thymidine. For the G_2 blockage and mitotic delay the changes of the mitotic index have generally been used. In addition to these methods, during the last years, rapid-flow cytometry has been used to investigate changes in the distribution of cells in the cell cycle (GÖHDE 1973, KAL 1973, RAJU et coll. 1974, 1980, LINDEN et coll. 1975, KÖNIG et coll. 1975, TRIBUKAIT 1975, FIDORRA & LINDEN 1977, SCHLAG et coll. 1978, LINDMO & PETTERSEN 1979, LÜCKE-HUHLE et coll. 1979, 1982, KÖNIG & BAISCH 1980, JUNG et coll. 1980, 1981, ZYWIETZ & JUNG 1980, TRIBUKAIT et coll. 1981, BECK et coll. 1981, LÜCKE-HUHLE 1982).

In the present investigation, the distribution of cells in the various parts of the cell cycle, obtained by flow cytometry, and the mitotic index were analysed following irradiation in the dose range of 1.75 to 8 Gy. These results have been combined with the analysis of the total number of cells, a relatively simple matter to follow the flow of cells through the cell cycle.

The results have been given in terms of the absolute values for the outflow rate of cells from the compartments, the outflow rate in relation to the pool size and also the difference between inflow and outflow rates related to the pool size. Finally, the duration of the various parts of the cell cycle and the total cell cycle duration were determined.

The mitotic delay time showed a dose dependent linear increase in the duration with a value of about

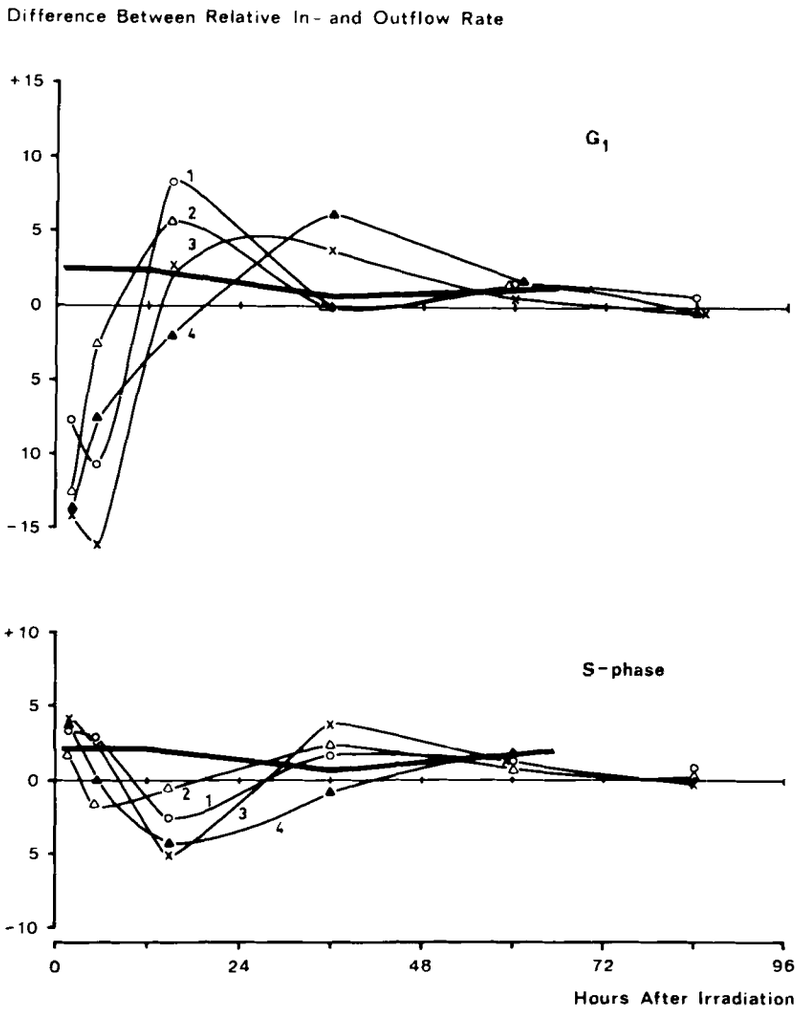

Fig. 8. Difference between in- and outflow of cells per hour as a percentage of the pool size after irradiation doses of 1.75 (1=○), 2.5 (2=△), 5 (3=×) and 8 (4=▲) Gy. Values for unirradiated cells are indicated by solid lines.

4 h per Gy (Fig. 3). The delay time was measured as the time between the mid-points of the descending and ascending mitotic index curves. Since the 8 Gy values did not reach the control levels the extrapolated value was used. The mitotic delay time as defined in this way cannot be compared directly with the mitotic delay measured by the appearance of the first mitotic cell after irradiation. This appearance is influenced by the number of cells measured and requires a large number of observations at various times after irradiation. In such a type of experiments values of about 1.7 h per Gy have been found in Ehrlich ascites tumour cells irradiated in vivo (KIM & EVANS 1964). The overshoot of the mitotic index and an oscillating behaviour have also been found in other cell systems which may indicate that the mitotic index is under the control of regulating influences. In the present experiments, in contrast to most other investigations, the mitotic index did not reach the control level following the relatively low dose of 8 Gy.

The accumulation rate of the M and G_1 cells after release of the mitotic division delay had a linear decrease with dose (Fig. 4). This deceleration in the accumulation rate is generally due to a decrease in the inflow rate. The G_2 blockage is the most evident event in the radiation effects on the cell proliferation. It expresses radiation induced cellular changes which are mostly acquired in parts of the cell cycle other than G_2 but which do not inhibit the ability of the cells to pass through these other stages. In the present experiments a continuously long-lasting par-

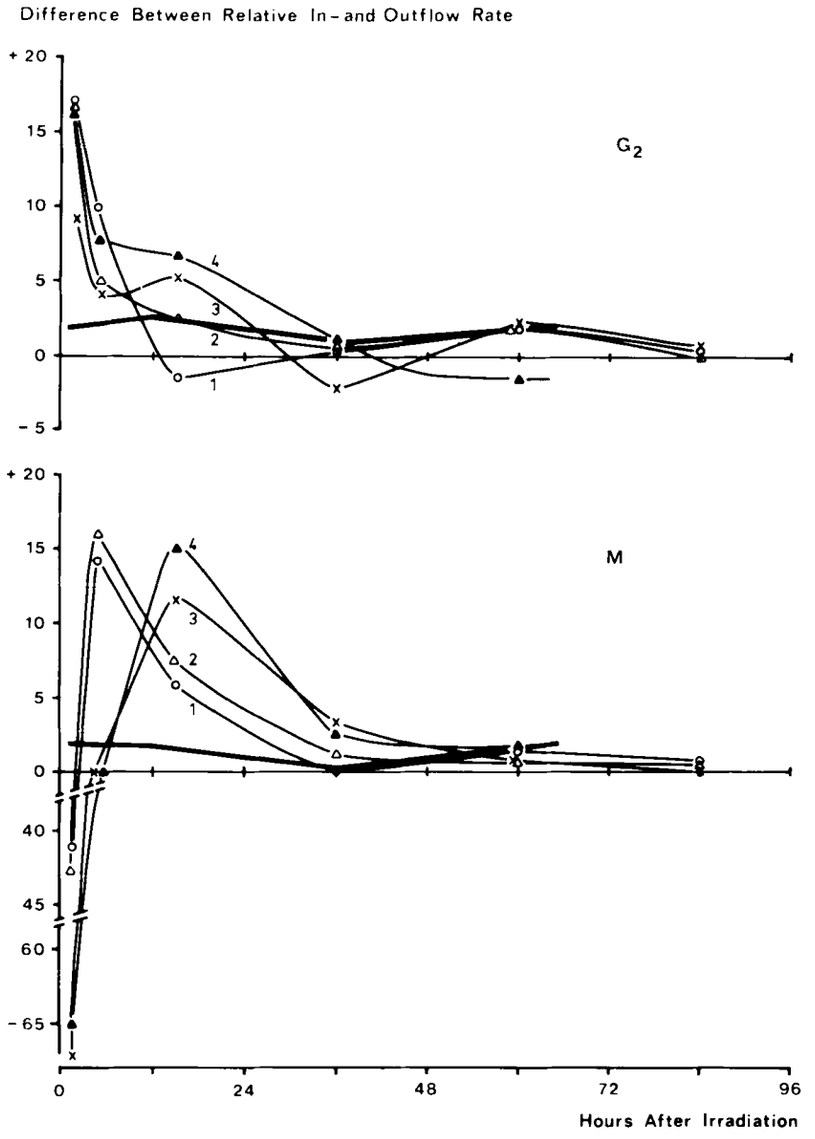

Fig. 8

tial G_2 blockage was found, previously described in detail (CAO et coll. 1982a). From this continuous partial blockage the early G_2 blockage up to about 72 h was demarcated by extrapolating the decrease in the cell number-time curve to the control level. Based on the cell number-time curves, a linear increase with dose was found for the duration of the early G_2 blockage (Fig. 3). If the maximum proportion of cells in G_2 was adopted as a measure for the extent of the G_2 blockage, as many authors have done, a maximum was reached already after a dose of 5 Gy at 24 h, at which time about 60 per cent of the cells were found to be in G_2. This value is of the same order of magnitude as that found in cultured cells after low LET ^{60}Co irradiation with the same dose (SCHLAG et coll., LÜCKE-HUHLE et coll. 1982), while in solid tumours lower peak values have been reported (LINDEN et coll., ZYWIETZ & JUNG). In experiments on Bp8 sarcoma cells growing in a solid form and locally irradiated with 5 Gy maximum values of about 30 per cent were found (HUANG et coll. 1982). Since the relationship between the inflow rate of cells into G_2 and the outflow rate of cells from G_2 determines the extent of the G_2 blockage, differences in the cell cycle characteristics may be partly responsible for these deviations in various tumours. Comparing the relationship between the inflow and outflow rates in the present experiment,

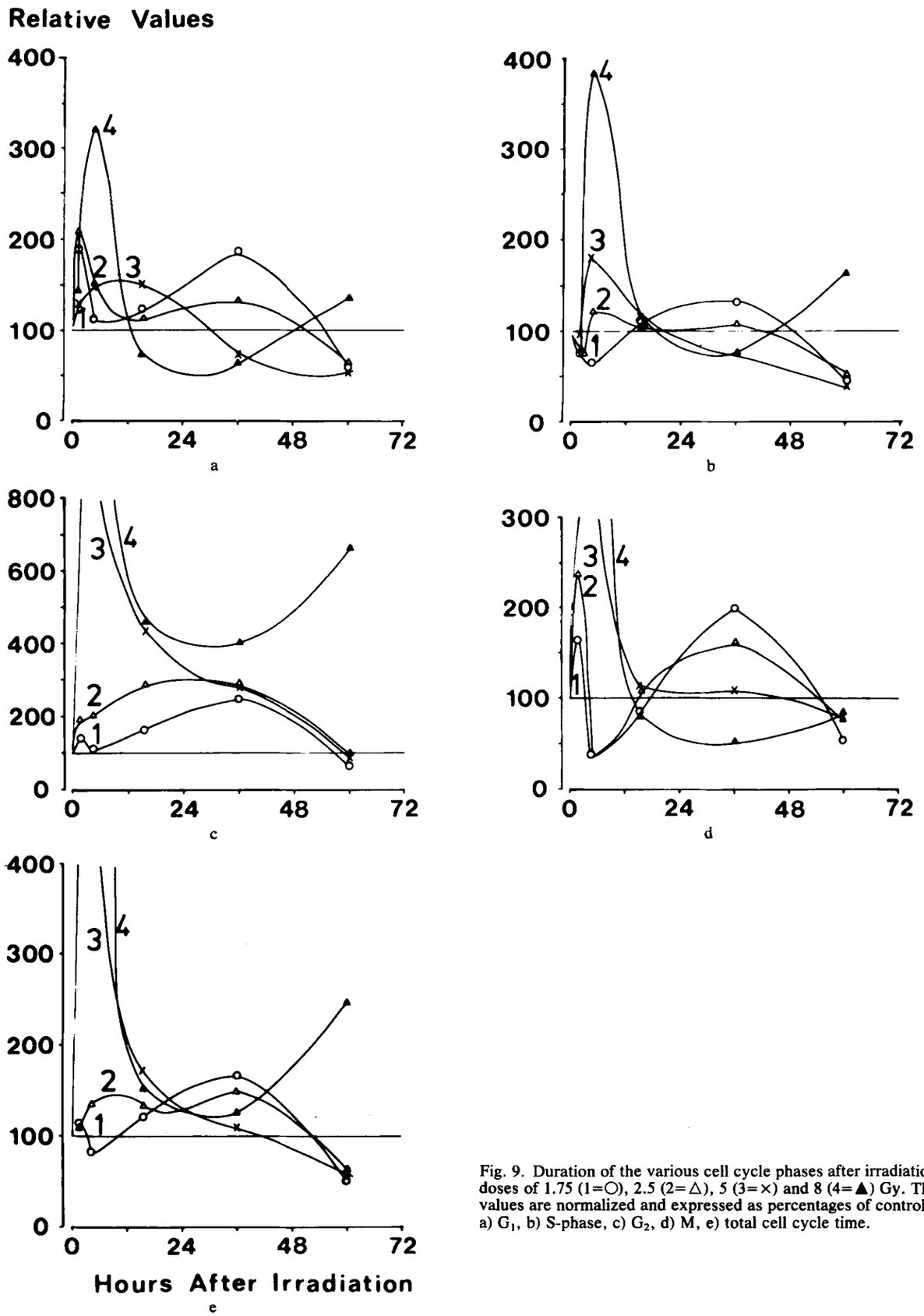

Fig. 9. Duration of the various cell cycle phases after irradiation doses of 1.75 (1=○), 2.5 (2=△), 5 (3=×) and 8 (4=▲) Gy. The values are normalized and expressed as percentages of controls. a) G_1, b) S-phase, c) G_2, d) M, e) total cell cycle time.

it is found that the reduced outflow rate is responsible for the increase in the extent of the G_2 blockage with dose.

For the rate of outflow from G_2 and M, after release of the G_2 blockage, a dose dependent exponential decrease with a shoulder was found (Fig. 6). Such types of dose response curves are generally found for the clonogenic properties of cells following low LET irradiation and have also been found for the Bp8 sarcoma with a D_0 of about 1 Gy and a n-value of about 1.2 (TRIBUKAIT et coll. 1982). The similarity of these two dose response curves may suggest some connection between G_2 blockage and cell survival. Using as another end point the total number of cells at the plateau phase of the growth curves, a shoulder curve is also found with, however, the considerably higher D_0 value of about 6.5 Gy (CAO et coll. 1982 a). Whether the radiation induced changes responsible for the G_2 blockage and cell survival are linked to each other is, however, only the subject of speculation.

The calculations on the duration of the various parts of the cell cycle are based on the mean values of the inflow and outflow rates divided by the pool size. If non-proliferating cells are present in a pool, the calculated durations are no longer the real ones. Since at around 15 hours following 8 Gy the G_1 compartment is almost completely empty, the number of non-proliferating G_1 cells is apparently very low. On the other hand, the long-lasting increase in the number of G_2 cells after irradiation may indicate non-proliferating cells in G_2.

Another precondition for correct estimation of the phase durations is that the age distribution of the cells in the pools of the compartments is homogeneous. This may not be the case, e.g. in the G_1 compartment at some times after release of the mitotic delay.

Finally, in these calculations the cell loss is not taken in account. According to total body measurements after prelabelling Bp8 sarcoma cells with ^{125}I-deoxyuridine the cell loss up to 3 days after irradiation is the same as in unirradiated animals and is about 5 per cent per day (KANEKO & TRIBUKAIT, unpublished).

In addition to a complete blockage of M and G_2 after 5 and 8 Gy with an infinite prolongation during the first few hours the calculated changes in the duration of the various parts of the cell cycle show a transient increase in the duration of G_1, S-phase and M. The prolongation of the S-phase due to a reduction in the rate of DNA synthesis has previously been described also in ascites tumuor cells (KIM & EVANS, TRIBUKAIT), and has later been described in detail in synchronized cell populations using sequential cellular DNA measurements in parallel with thymidine incorporation (KÖNIG et coll. 1975, LINDEMO & PETTERSEN, KÖNIG & BAISCH). Previously, an unchanged duration of G_1 has been found in rapid proliferating cell systems, while in non-dividing cells stimulated to divide a depression occurred (for review see OKADA). A G_1-delay has recently, however, also been found in rapid growing synchronized cell systems irradiated in the early G_1 (GERNER 1977, KÖNIG & BAISCH). The prolongation of M at the early time 0 to 3 h after irradiation in the present experiments is due to the relative decrease in the inflow rate; it can be assumed that due to the short duration of the mitosis the ages of cells in the pool rapidly reach a homogeneous distribution. In between 3 and 6 h the duration is then about half the normal value; during this period the pool size has decreased to about one third of the normal value and despite nearly equal inflow and outflow rates the duration decreases due to the small pool size. The increase in the duration found in G_1 and M during the 24 to 48 h period following 1.75 Gy is mainly due to the increase in pool size at this time.

The total cell cycle time up to 36 h is generally prolonged but is subsequently normalized except after 8 Gy. The prolongation is in accordance with previous findings in ascites tumours using the method of per cent labelled mitosis (KIM & EVANS, FRINDEL et coll. 1970).

In conclusion, it has been shown that the sequential determination of the total number of cells together with the analysis of the proportion of cells in the compartments of the cell cycle in principal enables analysis of the inflow and outflow rates of the cells from the various compartments, of the size of compartments and also of the cell flow through the compartments. These methods have been adopted after pertubation of the cell proliferation by irradiation.

SUMMARY

Bp8 ascites sarcoma cells growing in vivo were whole body irradiated with doses of 1.75 to 8 Gy. The inflow and outflow rates of cells in the various compartments of the cell cycle were estimated on the basis of sequential analysis of the total number of cells and from the proportion of cells in G_1, S-phase, G_2 and M. The flow through the

compartments was calculated from these data and from the sizes of the cell pools. The durations of the mitotic delay, the G_1-depletion and the early G_2 blockage were linearly related to the dose. After release of the mitotic division delay the accumulation rate of cells in M and G_1 decreased linearly with the dose; for G_2, plateau values for the accumulation rate were found after 2.5 Gy. The outflow rates from G_2 and M after release of the G_2 blockage showed a shoulder type of dose-response with a D_0 of 2.5 Gy and a n value of 1.5. In addition to an increase in the duration of G_2, generally responsible for the increase in the total cell cycle time at about 24 hours after irradiation and an increase in the duration of the S-phase up to 12 hours after irradiation, an early increase in the duration of G_1 and M was observed.

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