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79

Recovery response of dividing cells in the thymus of whole-body gamma-irradiated mice

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Mice were irradiated with different doses of gamma-rays 30 min after the administration of ^{32}P–orthophosphate. The dose–response curves determined at 72 hours after exposure showed an inflection point in the total activity present in the DNA in thymus and spleen. In the low dose-range, the dose–response curves have $D_0=55$ rad ($n=2{\cdot}5$) for thymus and $D_0=95$ rad ($n=2{\cdot}5$) for the spleen. Thirty minutes after the administration of ^{32}P-orthophosphate, the dividing cells from thymus were partially synchronized by the administration of 80 mg per kg body-weight hydroxyurea. At different time-intervals, the mice were irradiated with 80 rad, and the total activity of DNA was determined at 72 hours after synchronization. A significant maximum of recovery was found at 5 hours (S phase) after the administration of hydroxyurea. In similar conditions, the dose–response curves corresponding to the G_1, S and M phase of the division cycle were also determined. The synchronization of dividing cells induced by hydroxyurea failed in the spleen.

1. Introduction

The recovery of dividing cells from radiation injury has been demonstrated in different cellular systems (Elkind and Whitmore 1967), including bone-marrow (McCulloch and Till 1962, 1964) and splenic lymphocytes (Okada 1970). However, in early investigations, it was found that thymic lymphocytes were unable to recover from radiation damage (Jackson, Christensen and Bistline 1969, Jackson and and Christensen 1972). It was concluded that the mechanism responsible for thymus cell-death is radiation-induced interphase death followed by cell lysis (Jackson, Christensen and Forkey 1968, Okada 1970). More recently, from dose-fractionation studies, it was suggested that the mitotically-active cells from thymus can recover from radiation injury (Suciu, Uray and Abraham 1975). As an extension of this study, the present work was undertaken to determine the effect of whole-body irradiation on the dividing cells from thymus and spleen, previously labelled by the administration of ^{32}P-orthophosphate. The retention of ^{32}P-DNA in the thymus and spleen was taken as a measure of both the activity of DNA synthesis and the loss of dividing cells. The radiation response of dividing cells from thymus and spleen was also investigated after partial synchrony induced by hydroxyurea, an S-killing and G_1/S blocking agent (Brown 1975). The results support the assumption that the mitotically-active cells from thymus and spleen are capable of recovery from radiation injury without the loss of reproductive integrity.

2. Materials and methods

2.1. *Radioactive labelling*

Male mice of the NMRI strain weighing 28 ± 2 g were injected intraperitoneally with 25 μCi of ^{32}P-orthophosphate (6 Ci per mmole, IFA

Bucharest, Romania) or 25 μCi of ^{3}H–thymidine (5 Ci per mmole, Amersham, England).

2.2. *Irradiation conditions*

Mice were whole-body irradiated in plastic cages by a ^{60}Co therapeutic unit (Theratron 80) (FSD = 80 cm, 100 rad per min). The dose-rate was measured with a Siemens universal dosimeter. The irradiated and control mice were given a standard diet.

2.3. *DNA determination*

Thymus and spleen were homogenized in cold 0·14 N NaCl with a Potter–Elvehjem homogenizer. The homogenate was treated with a solution of KOH to a final concentration of 0·3 N, and the samples were incubated for 24 hours at 37°C. Perchloric acid was added to a final concentration of 0·2 N, and the samples were centrifuged for 10 min at 1400 × g. The supernatant fluid was discarded; and the sediment was washed in 0·2 N $HClO_4$ and centrifuged for 10 min at 1400 × g. The sediment was hydrolysed in 0·2 N $HClO_4$ for 15 min at 100°C. Fractions of hydrolysate were used for the diphenylamine colour reaction (De France and Le Pecq 1961) and the determination of radioactivity.

2.4. *Radioactive measurement*

Fractions of hydrolysate were dried on filter-paper discs, and the ^{32}P-radioactivity was counted with a mica end-window Geiger–Muller counter (Type VAZ) connected to a VA–M–16D Vakutronik counting system. The ^{3}H-thymidine radioactivity was counted in a liquid scintillation spectrometer (Betaszint BF–5003), as previously described (Suciu *et al.* 1975). From these measurements, the total activity (c.p.m. per DNA per organ) and specific activity (c.p.m. per mg DNA) of DNA in thymus and spleen were determined. The values given in figures are means of the results obtained from 8 to 16 mice ($\pm$SEM). Two hours after the administration of 25 μCi per mouse ^{3}H-thymidine the specific activity was 16·2 $\pm$ 1·1 mμCi per mg DNA in thymus and 54·0 $\pm$ 3·6 mμCi per mg DNA in the spleen. In similar conditions, after the administration of ^{32}P-orthophosphate the specific activity in thymus was 4·1 $\pm$ 0·3 mμCi per mg DNA and 7·2 $\pm$ 0·7 mμCi per mg DNA in the spleen. These values were obtained after correction of the quenching effect.

3. Results

3.1. *DNA labelling*

After the administration of ^{3}H-thymidine or ^{32}P-orthophosphate, the specific activity of DNA was determined at different time-intervals in thymus and spleen (figure 1). As has previously been shown (Nygaard and Potter 1959, Pierucci 1967), the incorporation of labelled thymidine into thymus and spleen is almost complete within 2 hours after injection. In the following 6 days, a continuous decrease of the specific activity of DNA can be observed in both thymus and spleen (figure 1) (Nygaard and Potter 1960, Sanders, Dalrymple and Robinette 1964). It was assumed that reincorporation of labelled thymidine released through breakdown of cells is negligible (Nygaard and Potter 1960). Thus, the loss of labelled DNA within the period of several days after labelling has been equated with the loss of labelled cells from the tissue (Nygaard and Potter 1960,

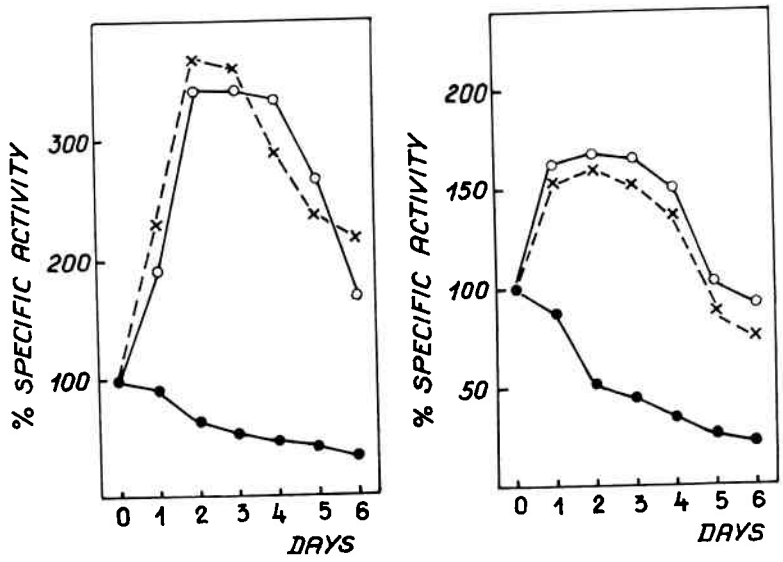

Figure 1. Time-course of the specific activity of DNA in thymus (left) and spleen (right) after administration of ^{3}H-thymidine (●) or ^{32}P-orthophosphate (○) (×). DNA analysis was performed as described in § 2.3. (○) or according to Schmidt and Thannhauser (1945) (×). Results expressed as percentage of specific activity values at 2 hours after administration of the label. SEM was less than 14 per cent.

Gerber, Gerber, Altman and Hempelmann 1963, Sanders *et al.* 1964, Suciu *et al.* 1975). However, the assumption that labelled thymidine reincorporation is negligible in thymus and spleen is probably not strictly correct, since the loss of ^{125}I–IUdR-labelled DNA is faster still, where no reincorporation is possible (Fox and Prusoff 1965, Myers and Feinendegen 1975). On the other hand, the specific activity of ^{32}P-DNA increases up to 2 days after administration of the label (figure 1). In principle, the *in vivo* continuous incorporation of ^{32}P into

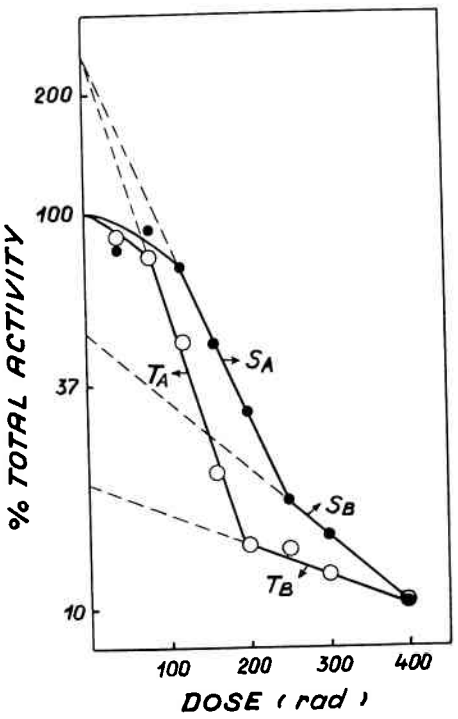

Figure 2. Dose–response curves of the total activity of DNA in thymus (○) and spleen (●) at 72 hours after irradiation. ^{32}P-orthophosphate was administered 30 min before irradiation. Results expressed as percentage of total activity values of unirradiated controls at 72 hours after administration of the label. SEM was less than 9 per cent.

DNA can be compared with the *in vitro* method of continuous labelling by the addition of labelled thymidine into the culture medium (Mak and Till 1963, Yamada and Puck 1961). As the purpose of this study is to investigate the radiation-recovery response of dividing cells in thymus and spleen, we used the ^{32}P-orthophosphate-labelling of DNA as a measure of both the continuous activity of DNA synthesis and the loss of previously-labelled cells (figures 2, 3, 5 and 6). However, further work is necessary to establish the mechanism of continuous labelling of DNA after the *in vivo* administration of ^{32}P-orthophosphate.

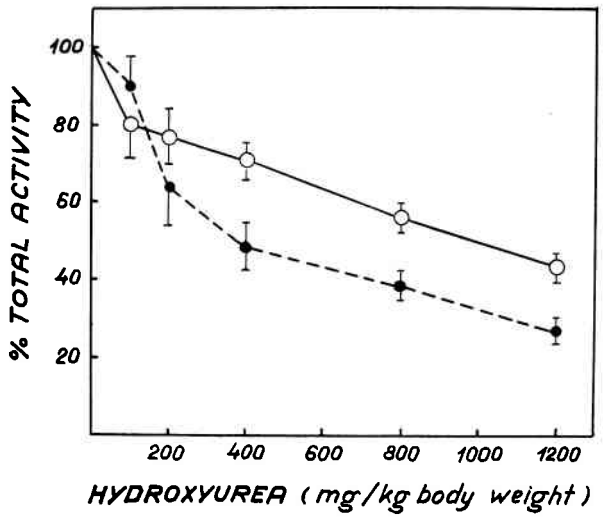

Figure 3. Total activity of DNA in thymus (●) and spleen (○) at 24 hours after administration of different doses of hydroxyurea. ^{32}P-orthophosphate was administered 30 min before injection of hydroxyurea. Results expressed as percentage of total activity of DNA at 24 hours after administration of the label.

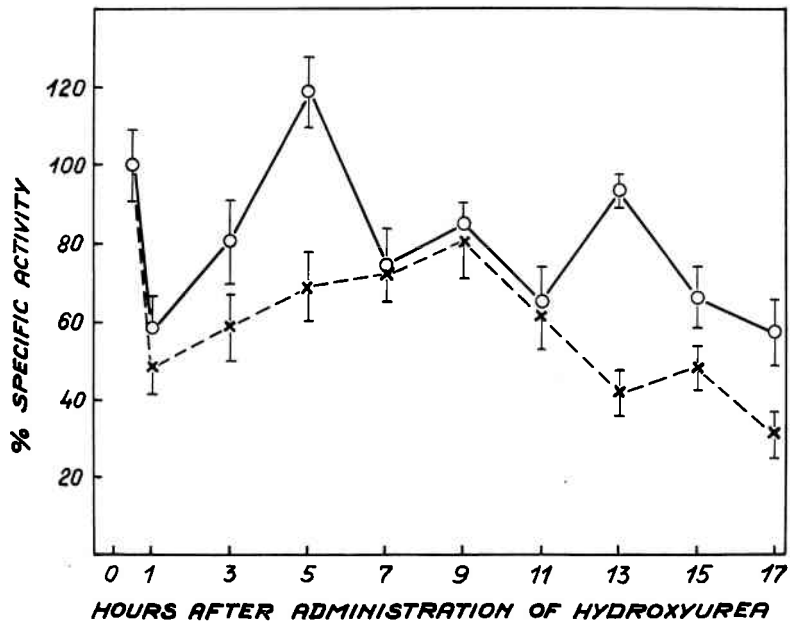

Figure 4. Specific activity of DNA in thymus (○) and spleen (×) at different time-intervals after administration of 80 mg per kg body-weight hydroxyurea. Animals were killed 30 min after administration of ^{32}P-orthophosphate. Results expressed as percentage of specific activity values of untreated controls at 30 min after administration of the label.

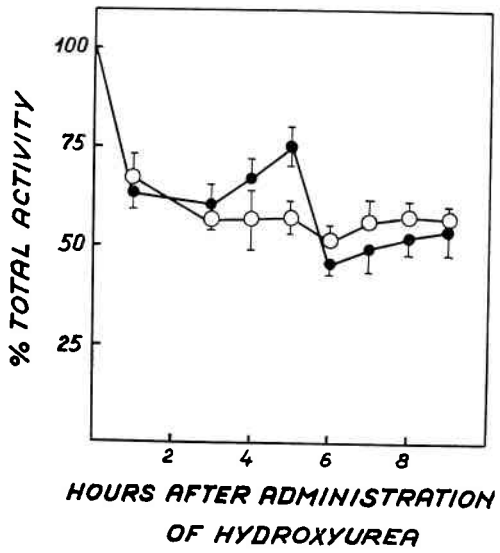

Figure 5. Total activity of DNA in thymus (●) and spleen (○) at 72 hours after administration of 80 mg per kg body-weight hydroxyurea. ^{32}P-orthophosphate was administered 30 min before injection of hydroxyurea. At different time-intervals the animals received 80 rad. Control group was irradiated with 80 rad at 30 min after administration of the label. Results expressed as percentage of total activity DNA in the control group.

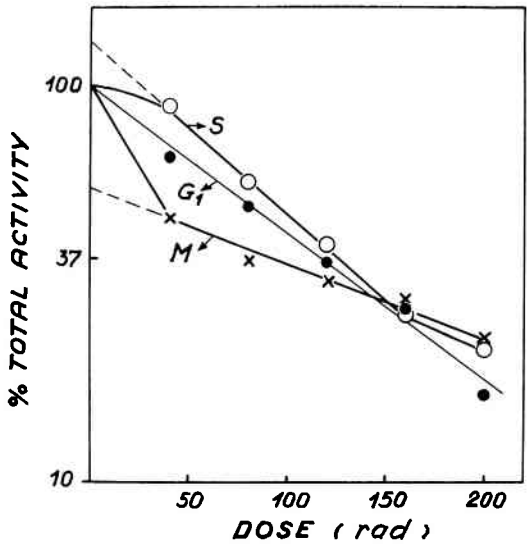

Figure 6. Dose–response curves of the total activity of DNA in thymus. ^{32}P-orthophosphate was administered 30 min before injection of 80 mg per kg body-weight hydroxyurea. Animals were given different doses of radiation at 1 hour (●) (G_1), 5 hours (○) (S) and 6 hours (×) (M) after administration of hydroxyurea. Control group was unirradiated. Mice were killed 72 hours after administration of hydroxyurea. Results expressed as percentage of total activity of DNA in the control group. SEM was less than 8 per cent.

3.2. *Dose–response curves*

Mice were whole-body irradiated with different doses 30 min after the administration of ^{32}P-orthophosphate. The total activity of DNA was determined in thymus and spleen 72 hours after irradiation. The dose–response curves have a shoulder in the low dose-range corresponding to S_A and T_A (figure 2). The inflection point (Elkind and Whitmore 1967) indicates that a fraction of the thymus and spleen population is represented by cells with an increased radio-resistance in the high dose-range (curve T_B and S_B from figure 2). The

parameters of the dose–response curves are included in the table. The survival parameters of spleen cells as determined by other methods are $\tilde{D}_0$ from 47 to 78 rad and n from 1·8 to 3·3 (Okada 1970).

Curve	$\tilde{D}_0$ value (rad)	Extrapolation number $\tilde{n}$
T_A	55	2·5
T_B	300†	
S_A	95	2·5
S_B	250†	

Significance of T_A, T_B, S_A and S_B is given in figure 2 and § 3.2.
† Determined by extrapolation (figure 2).

Parameters of the dose–response curves shown in figure 2.

3.3. *Effect of hydroxyurea on the dividing cells from thymus and spleen*

The depletion of ^{32}P-DNA in thymus and spleen at 24 hours after administration of different doses of hydroxyurea is shown in figure 3. It was demonstrated that the S-killing effect of hydroxyurea is common for all cell systems with high rates of cellular proliferation (Sinclair 1965, Philips, Sternberg, Schwartz, Cronin, Sodergren and Vidal 1967, Withers 1975). As the utility of hydroxyurea in this investigation is only to induce the synchrony of dividing cells, we decided to limit the dose of drug to 80 mg per kg body-weight (figures 4–6). The effect of this dose on the incorporation of ^{32}P-orthophosphate into the thymus and spleen DNA is shown in figure 4. The pulse labelling experiment (figure 4) suggests the G_1 block induced by hydroxyurea, as determined by the decrease of DNA synthesis few hous after administration of the drug.

3.4. *Radiosensitization of dividing cells in thymus and spleen after administration of hydroxyurea*

Mice were given 80 mg per kg body-weight hydroxyurea 30 min after the administration of ^{32}P-orthophosphate. At different time-intervals the animals were irradiated with 80 rad (figure 5). The total activity of DNA was determined in thymus and spleen at 72 hours after synchronization. If irradiation was carried out at 5 hours after administration of hydroxyurea the radioactivity of thymus DNA was significantly increased ($p < 0{\cdot}05$) compared with the value determined for the animals irradiated 6 hours after synchronization. The recovery maximum (figure 5) corresponds to the maximum of DNA synthesis (figure 4).

The recovery effect was absent in the spleen (figure 5). Accordingly, DNA synthesis was decreased for a relatively long period after the administration of hydroxyurea (figure 4).

The preceding data allowed the determination of the dose–response curves shown in figure 6. The animals received ^{32}P-orthophosphate 30 min before the administration of 80 mg per kg body-weight hydroxyurea. Irradiation was carried out at 1 hour, 5 hours and 6 hours after the partial synchronization. The

total activity of thymus DNA was determined 72 hours after the administration of hydroxyurea. As the mean cell-cycle time for dividing thymocytes was found to be in the range of 6 to 8 hours (Metcalf and Wiadrowski 1966), it is probable that the time of irradiation corresponds to the G_1 (1 hour), S (5 hours) and G_2 and M phases (6 hours) of the division cycle. The G_1 curve (figure 6) was exponentially related to dose, indicating that no recovery occurs for this cell population ($D_0 = 115$ rad). For the S curve we found $D_0 = 85$ rad and $n = 1{\cdot}2$. If irradiation was performed at 6 hours (G_2 and M phase) after the administration of hydroxyurea, about 50 per cent of the total activity of DNA was lost at a dose of 40 rad.

4. Discussion

The data reported in this work are based on the assumption that the depletion of the previously-labelled DNA in the thymus and spleen of irradiated animals corresponds to the loss of cells with labelled DNA (Nygaard and Potter 1960, Sanders *et al.* 1964, Gerber *et al.* 1963, Suciu *et al.* 1975).

The thymic population is represented by the mitotically-active medium and large thymocytes and the non-dividing small thymocytes (Borum 1973, Metcalf and Wiadrowski 1966). The mean cell-cycle time of dividing thymocytes was estimated to be in the range of 6 to 8 hours. The dividing fraction of cells represents about 10 per cent from the whole lymphoid population. The medium and large thymocytes are continuously transformed into non-dividing small thymocytes. The process of cell renewal of the small size cells requires about 3 days (Metcalf and Wiadrowski 1966). As ^{32}P-orthophosphate is continuously incorporated (figure 1) but only into the DNA of the dividing fraction of cells (Ord and Stocken 1956), the labelling of DNA in the whole population requires about 2 or 3 days. The incorporation of labelled precursors into the DNA of thymus cells by other routes than cell division is improbable (Borum 1973). The dose–response curves determined at 72 hours after irradiation (figure 2 and table) support the assumption that in the low dose-range the dividing cells from thymus are capable of recovery from radiation injury. Due to the short cell-cycle time, it is probable that 72 hours after irradiation, when the process of cell depletion is terminated (Jackson and Christensen 1972), the remaining fraction of thymic cells represent surviving cells (Fabrikant 1972). The experiments shown in figure 4 suggest that the G_1/S blocking effect of 80 mg per kg body-weight hydroxyurea is limited to about 3 hours. Then, the DNA synthesis increases and the radiation recovery response is clearly evident (figures 5 and 6). The low dose-range of the dose-response curves (figure 6) corresponding to a representative fraction of *in vivo* synchronized thymus cells indicates that the S phase of the division cycle is the most resistant to radiation. These findings are in agreement with previous observations on the radiosensitivity and recovery response of other mammalian cell systems after synchrony induced by hydroxyurea (Brown 1975).

In the splenic lymphoid population both the large and small size cells are capable of division (Fliedner, Kesse, Cronkite and Robertson 1964, Hanna 1964, Post and Hoffman 1970). On the other hand, it was suggested that the spleen lymphoid cells consist of at least two fractions with different levels of radiosensitivity (Nygaard and Potter 1960), Gerber and Altman 1970, Raff 1973, Streffer 1974), and probable different cell-cycle times (Fliedner *et al.* 1964,

Hanna 1964, Post and Hoffman 1970). It is therefore conceivable that the attempt to synchronize the population of dividing cells failed (figures 4 and 5). The radiation-recovery response of the lymphoid population from spleen is represented by the low dose-range of the dose–response curve reported in figure 2. It may be assumed that for both thymus and spleen the high dose-range of the dose–response curves (figure 2) reflects the radiation response of some non-lymphoid cellular systems.

In conclusion, our data support the view that the thymic and splenic dividing cells are capable of recovery from radiation injury. However, as the recovery process was studied almost exclusively in cell-survival experiments (Elkind and Whitmore 1967, Okada 1970) our results appear as indirect evidences.

Acknowledgment

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Les souris ont été irradiées avec des doses différentes, 30 minutes après l'administration du ^{32}P-orthophosphate. Conformément aux analyses faites 72 heures après l'exposition, les courbes dose–réponse de l'activité totale de l'ADN dans le thymus et la rate présentaient un point d'inflexion. Dans le domaine des doses basses les courbes dose–réponse ont $D_0 = 55$ rad ($n = 2{,}5$) pour le thymus et $D_0 = 95$ rad ($n = 2{,}5$) pour la rate. Dans une autre expérience, 30 min après l'administration du ^{32}P-orthophosphate, les cellules en division ont été partiellement synchronisées par l'administration de 80 mg per kg corps. hydroxyurée. A des intervalles de temps différents les souris ont été irradiées avec 80 rad et 72 heures après la synchronisation on a determiné l'activité totale de l'ADN. On à trouvé un maximum de rétablissement 5 heures (phase S) après l'administration de l'hydroxyurée. Dans des conditions similaires, on a déterminé aussi les courbes dose–réponse correspondant à la phase G_1, S et M du cycle cellulaire. La synchronisation des cellules en division par la hydroxyurée n'a pas eu lieu dans la rate.

Mäuse wurden 30 min nach Verabreichung von ^{32}P-Ortophosphat mit verschiedenen Dosen bestrahlt. 72 Stunden nach der Bestrahlung konnte festgestellt werden, dass die Dosis-Effekt-Kurven der DNS-Gesamtaktivität in Thymus und Milz einen Inflexionspunkt aufweisen. Im Bereich der niedrigen Dosen zeigen die Dosis-Effekt-Kurven $D_0 = 55$ rad ($n = 2{,}5$) für Thymus und $D_0 = 95$ rad ($n = 2{,}5$) für Milz. In einem anderen Experiment wurden 30 min nach der Verabreichung von ^{32}P-Ortophosphat die sich teilenden Zellen aus Thymus durch Gaben von 80 mg Hydroxyharnstoff/kg Körpergewicht partiell synchronisiert. In verschiedenen Zeitabständen wurden die Mäuse mit 80 rad bestrahlt und 72 Stunden nach der Synchronisierung die Gesamtaktivität der DNS bestimmt. Ein bedeutsames Erholungsmaximum bei 5 Stunden (Phase S) nach der Verabreichung von Hydrohyharnstoff wurde gefunden. Unter gleichartigen Bedingungen wurden auch die Dosis-Effekt-kurven, für die Phasen G_1, S und M des Zellzyklus bestimmt. In der Milz fand die Synchronisierung durch Hydroxyharnstoff der sich teilenden Zellen nicht statt.

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