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ORGANIZATION OF THE DEOXYRIBONUCLEIC ACID REPLICATING SYSTEM IN MAMMALIAN CELLS AS REVEALED BY THE USE OF X-RADIATION AND BROMURACIL DEOXYRIBOSIDE

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IN previous work[1], the action of X-radiation on synthesis of deoxyribonucleic acid (DNA) was shown to result in a dose-dependent depression of rate, as measured by incorporation of tritium-labelled thymidine. The dose-response curve consisted of two components, one showing a very steep slope, the other a much shallower one. In those investigations incorporation of the tracer material was allowed to occur for 5–6 h, so that not only cells in the process of DNA synthesis (*S* period) at the time of irradiation incorporated the tracer, but also those cells in the pre-DNA synthesis (G_1) phase during the irradiation that entered *S* during the tracer incubation. In most of the work reported here, incubations for 1 h with tracers were used, so that predominantly *S* cells were labelled, and the contribution from cells in G_1 can be ignored.

Among these experiments are ones in which the analogue of thymidine, bromuracil deoxyriboside (BUdR), was incorporated into the DNA of the cells before the irradiation. The results of this treatment have suggested an interpretation of the dose-dependent depression of DNA synthesis rate by X-radiation and also permitted us to put forth a hypothesis concerning the sub-chromosomal organization of DNA.

Two kinds of cell culture were used in these investigations, HeLa *S*3 and the Chinese hamster line, *DFAF*-33, kindly supplied by Dr. George Yerganian. The HeLa *S*3 culture was cultured routinely in Eagle's medium and transferred weekly; the *DFAF*-33 was cultured either in Eagle's or in the 'HU-15' medium of Elkind[2], and transferred twice a week. For experiments, HeLa *S*3 cells were transferred, in Eagle's medium, at about 2–4 × 10^4 cells/ml., into any of 3 different kinds of culture vessels: *T*-30 flasks (5-ml./flask), Leighton tubes (1-ml./flask), or square centrifugible flasks (Bellco Glass, Vineland, New Jersey, 3-ml./flask). *DFAF*-33 cells for experiments

were always transferred into Eagle's medium, using the same array of culture vessels: incubations of HeLa *S3* prior to the irradiations varied from 3 to 5 days; for *DFAF*-33 they were 2 or 3 days. In 3-day experiments, the media were changed on the second day; in 5-day experiments they were changed on the third day.

Irradiations were performed with two different units. The first, used in the earlier experiments, was a 4-m.amp, 250-kVp. unit, with no external filtering; the dose rate was 100 r./min. The other was a 20-m.amp, 300-kVp. unit, also operated without external filtering; the dose rate, measured inside a tissue culture vessel, was from 200 to 300 r./min, depending on the number of cultures irradiated. All irradiations occurred on a rotating turntable, under conditions of minimal scatter, at room temperature.

Immediately after irradiation, the cultures, including the sham-irradiated controls, were returned to the laboratory, the media on them removed, and Eagle's medium containing the tracer added. The tracer used in most of the work was tritiated thymidine at 1 μc./ml., but the total thymidine of the medium was changed according to experimental requirements by the addition of unlabelled thymidine. Tritium-labelled uridine at 1 c./mM and ^{14}C-guanine at 3 mc./mM were also used at various times. All compounds were obtained from New England Nuclear Corporation. After the 37° incubation period (generally 1 h with exceptions as indicated later), the medium was rapidly removed from all cultures, and the cells processed to determine the specific activity of DNA.

Three different methods for DNA extraction were used. One method, previously described by us[1], was essentially that of Ogur and Rosen[3] using perchloric acid to hydrolyse both RNA and DNA. The second was the Schmidt–Thannhauser method[4], using overnight incubation with 1 N sodium hydroxide at 37° to hydrolyse RNA and perchloric acid at 70° C for 1 h to release DNA. The third was an adaptation of the method of Scott *et al.*[5] which uses 1 N sodium hydroxide for 1 h at room temperature to obtain RNA, hydrolysis with perchloric acid at 60° C for only 7 min to obtain the DNA fraction. All methods gave qualitatively the same results, but the latter method gave the best reproducibility, that is, the lowest variability among similarly treated cultures. In all cases the DNA of each flask was estimated by reading the optical density of the DNA fraction at 267 mM, using a Beckman *DU* spectrophotometer and semi-micro (volume about 1·5 ml.) cuvettes (Pyrocell Corp., New York). The radioactivity of the fractions was determined by dissolving 0·5 or 1·0 ml. of the sample in 10 or 15 ml. of counting solution (13 g 2,5-diphenoloxazole, 0·25 g 1,4-*bis*-2-(5-phenyloxazolyl)-benzene and 130 g naphthalene in a mixture of 1 l. toluene, 1 l. dioxane and 600 ml. ethanol), and counting in a

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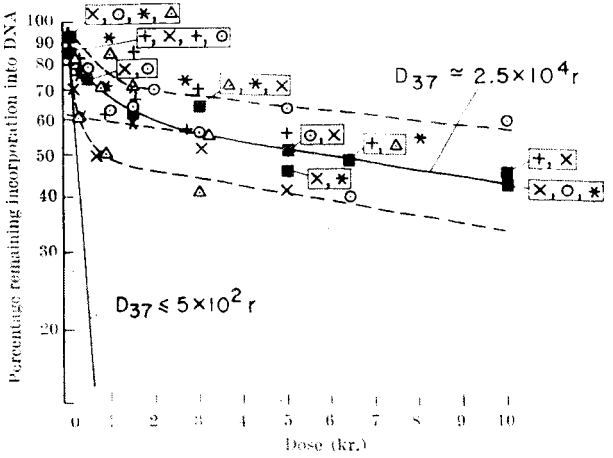

Fig. 1. Dose-response of HeLa S3 DNA synthesis to X-radiation. A composite of fourteen experiments, using tritiated thymidine of various specific activities. Each point is average of either two, three or four individual determinations. Data plotted as percentage of sham-irradiated controls, based on tritium counts per min per microgram DNA

Packard Tri-Carb liquid scintillation spectrometer. When tritium and carbon-14 were in the same samples, the efficiency of each isotope for each channel was determined using ^{3}H- and ^{14}C-toluene standards and the separate contributions of each isotope to the final counts calculated by means of simultaneous equations[1].

In experiments with bromuracil deoxyriboside (BUdR), experimental cultures were set up as usual; three days later the medium of one-half of the culture tubes was removed and replaced with Eagle's medium containing 5 μg/ml. BUdR ($1{\cdot}6 \times 10^{-5}$ M), and the medium of the other half of the tubes was replaced with Eagle's containing an equimolar amount of thymidine (4 μg/ml.). After two additional days' incubation, these media were removed, the cells washed once with Hanks's balanced salt solution and this solution was added to the flasks and gassed with 5 per cent carbon dioxide and 95 per cent air mixture. The flasks were then irradiated, returned immediately to the laboratory, the Hanks's balanced salt solution removed from them and replaced with Eagle's medium containing the radioisotope(s).

The results of 14 experiments using 1 μc./ml. tritiated thymidine as the tracer for DNA synthesis in HeLa *S*3 cells are plotted in Fig. 1. The concentration of thymidine varied from $1{\cdot}5 \times 10^{-5}$ to 4×10^{-2} M, that is, the specific activity of the tritiated thymidine in these experiments varied from 24 mc./mM to 6,700 mc./mM. There is no consistent effect of specific activity. The variability that is evident from the scatter is much more the result

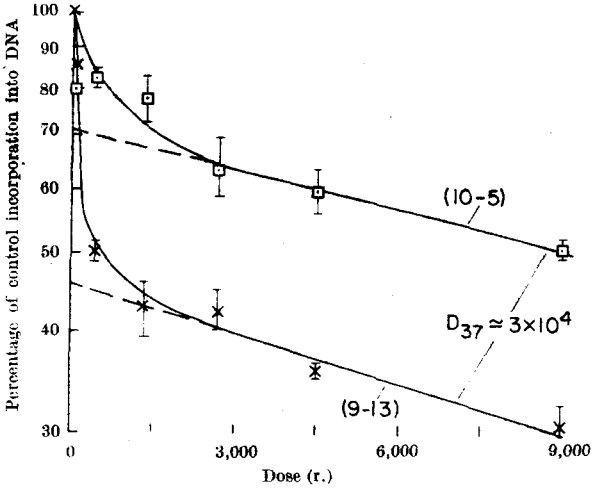

Fig. 2. Dose-response of *DFAF*-33 DNA synthesis to X-radiation. illustrating variation in participation of steep component

of dose-response differences between experiments than from heterogeneity within an experiment. The maximum and minimum total inhibitions are plotted by extrapolation to zero dose; it appears that the fraction of the total inhibition due to the steep component varies between roughly 25 and 55 per cent. Two experiments with *DFAF*-33, shown in Fig. 2, demonstrate a similar variability, which is comparable to that reported by Lajtha *et al.* for bone marrow in culture[6]. Results with low specific activity ^{14}C-guanine at 10 μg/ml., used in double-labelling experiments with tritiated thymidine, are practically identical with those obtained with tritiated thymidine, and assure that the steep component is not due to a release of pool materials that compete with tracer for sites in DNA.

The D_0 of the shallow component of the curve varies as much as two-fold if values of individual experiments are plotted. However, the range of values from about 1·8 to 4 × 10^4 r. affects the estimated size of the hypothetical target only by approximately two. On the other hand, although it is rather difficult to measure exactly the average D_0 of the steep component, it is so small, certainly less than 500 r., that the resulting target volume must be very large.

The apparent extent of depression of DNA synthesis by X-radiation increases as a function of time of incubation with tracer. This is illustrated in Table 1 and is similar to the results reported previously[1]. This observation indicates that cells that are in the G_1 compartment during

Table 1. Effect of Post-irradiation Incubation Time on the Radiation-depressed Incorporation of H^3TdR into DNA

Exp. No.	Dose (r.)	Specific activity c.p.m./μg DNA 1 h incubation	Depression (per cent)	3 or 4 h* incubation	Depression (per cent)
1	0	377	—	1,090	—
	100	342	9	808	26
	800	295	22	602	45
2	0	332	—	1,062	—
	500	274	17	634	42
	5,000	177	47	346	68

* Incubation time following irradiation in Exp. 1 was 4 h; in Exp. 2 it was 3 h.

Table 2. Effect of Low Doses of X-radiation on Uptake of Tritiated Thymidine into DNA of Thymidine-grown Cells and Bromuracil Deoxyriboside-grown Cells

Dose (r.)	Sp. act. of thymidine-grown cells	Change (per cent)		Sp. act. of BUdR-grown cells	Change (per cent)	
0 (control)	1,112 ± 44	—		678 ± 27	—	
52	1,008 ± 50	−9	−10·6*	685 ± 18	+1	+3·2*
162	980 ± 60	−12		716 ± 42	+5	

* Pooled means change in thymidine-grown cells differs significantly from that of BUdR-grown cells; $t_{(18\,d.f.)} = 3{\cdot}06$; $P_{(\bar{x}_{TdR} - \bar{x}_{BUdR})} < 0{\cdot}01$.

irradiation and enter the *S* period during the next 3–4 h are affected roughly the same, in terms of rate of DNA synthesis, as cells in *S* at the time of irradiation.

A typical result of the effect of BUdR incorporated into DNA on the dose-response of DNA synthesis is shown in Fig. 3; the dose-response curve shows an increased incorporation of isotope into DNA over control values at low doses, followed by a dose-response practically identical to that observed with thymidine-grown cells. In order to establish further the reality of this difference, an experiment was performed using 5 replicate flasks per treatment, with controls and only two low doses, 52 and 162 *r*. The results (Table 2) show that these small doses slightly but significantly depressed the incorporation into DNA of thymidine-grown cells, but had no effect on the BUdR-grown cells. It is important to note that the effect of BUdR in sham-control cultures is to very markedly decrease the rate of DNA synthesis. The enhanced incorporation of tritiated thymidine into DNA by low doses of X-radiation occurred in about one-half the experiments and, in every instance except one (among 10 experiments), the effect of BUdR was to decrease the extent of depression of incorporation by small doses. We have never observed enhanced incorporation under any other circumstances. At higher doses the results paralleled those of normally grown or thymidine-grown cells so that there is no apparent effect on the second component of the dose response. It is possible that a subtle effect exists in this area, but, if so, it is hidden in the variability of the response.

The two-component dose-response curve is similar to

those reported by Lajtha and co-workers for bone marrow in culture[6], for bone marrow and ascites tumour cells by Jasinska and Michalowski[7], and for rat thymus by Ord and Stocken[8]. A number of theories have been proposed to explain these results. In thymus, Ord and Stocken attribute the steep fall (corresponding to the steep or S_1 component of the two-part curve) to inhibition of nuclear phosphorylation, but this explanation, even if it were a general one (which it appears not to be), still does not explain the site of the damage. Although damage to an enzyme has been suggested[6], this seems highly unlikely in terms of target theory, which would indicate that the molecular weight of the hypothetical enzyme would be in the order of 10^9, clearly much greater than molecular weights of known enzymes.

Our results with BUdR lead us to an alternative explanation. This competitor of thymidine for DNA thymine sites depresses the rate of synthesis of DNA, and since it is no longer available for incorporation from the medium at the time of this inhibition, it is the BUdR in the DNA, and presumably in those areas acting as templates for new synthesis, that contributes to this rate depression. A small dose of X-radiation, in many instances, partially reverses this inhibition. We propose that the incorporated BUdR distorts the organization of a large organized component of the DNA replicating system that is necessary to maintain the maximal rate of replication of DNA. The size of this is estimated to be in the order of 10^9–10^{10}, since a small dose (500 r.) of X-rays can affect it. The effect of a single hit on the BUdR-substituted DNA is sometimes to make a new site available for synthesis so that a stimulation of rate may be observed. On the whole, however, the effect is to further the disorganization of this 'super molecule', and so larger doses result in depression of rate.

The concept of very-high-molecular-weight DNA is not new. The work of Davison[9], showing the effects of shear on molecular weight determinations, has stimulated a great deal of recent work in this area. In general, the concept has evolved that the molecular weight of native DNA is very much larger than the often-quoted $1–8 \times 10^6$ found in the older literature. The researches of Cairns[10] and of Kleinschmidt *et al.*[11] have led to the concept that all the DNA in a bacterium like *Escherichia coli* (at least all the DNA in one nucleus therein) is in the form of a single large molecule. Interestingly, the molecular weight of this DNA is about 10^9.

According to Lee and Puck[12], the average content of DNA of the HeLa cell is 17 pg, or a total molecular weight of DNA per cell of about 10^{13}. This is distributed, probably unequally, among 78 (on the average) chromosomes. Taking one of the smaller chromosomes to contain

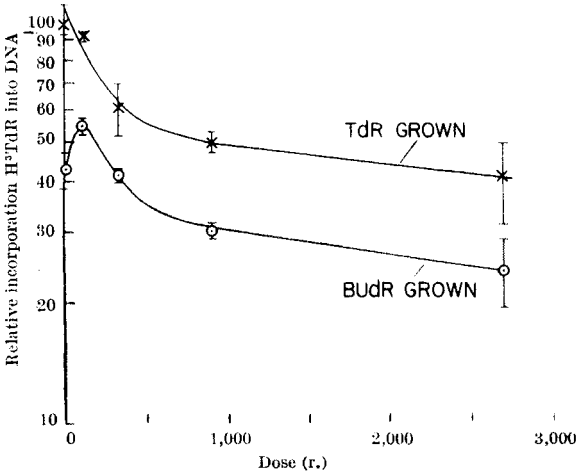

Fig. 3. Effect of bromuracil deoxyriboside (BUdR) on dose-response of HeLa *S*3 DNA synthesis to X-radiation. BUdR cultures grown in presence of 5 μg/ml. BUdR, controls in presence of 4 μg/ml. thymidine (TdR), for 48 h prior to irradiation. All data plotted relative to specific activity of TdR-grown control
(H^3TdR = tritiated thymidine)

roughly ½ 1/100 of the total DNA of the cell, its total DNA would be about 10^{11}. The target estimate of the large molecule of DNA involved in regulating rate of DNA synthesis is 10^9–10^{10}, so the chromosome would contain 10–100 of these components. That chromosomes do contain several 'replicating units' is borne out by autoradiographic investigations that have shown DNA synthesis occurring simultaneously at two or more sites in a chromosome, while other parts of it show no evidence of replication[13–15].

Even if the integrity of this large unit is completely destroyed (as by inactivation with X-rays) DNA synthesis continues, but at a considerably diminished rate (shallow component), and only very large doses of radiation, capable of inactivating that DNA acting as a 'template', can significantly depress the rate further. These results indicate that DNA of molecular weight of about 10^7 is 'activated' for replication at any one time.

Our interpretation is strengthened by the results of Lehnert and Okada[16], who have shown that an effect of X-radiation on DNA synthesis rate in nuclei of regenerating rat liver can be observed so long as protein is still in association with DNA, but no effect is found with purified DNA alone. Walwick and Main[17] have also reported no effect of ionizing radiation, up to 10,000 r., on the rate of DNA synthesis in a cell-free system using purified DNA as primer. These kinds of investigations point out the

limitations of *in vitro* biochemical methods, wherein maximum mixing of components and completely unorganized systems are used, as a means for determining effects on the highly ordered state of subcellular organization. It is probable that when purified 'primer' DNA is used in *in vitro* reactions it is at a much lower molecular weight than in the cell. Moreover, it is removed from restrictions on its reaction probability by being free of protein, now greatly implicated in the function of DNA as a template for RNA (particularly 'messenger' RNA) synthesis[18–20]. It is not surprising that ionizing radiation cannot reduce the efficiency of DNA as a primer under these conditions where there is at all times a maximum likelihood of its interaction with the enzyme and precursors. Indeed, if the action of ionizing radiation is primarily to reduce the size of the primer unit, it could conceivably take enough radiation to reduce the average molecular weight to somewhere less than 10,000, which is the molecular weight of yeast lactic dehydrogenase-associated DNA[21,22], shown to act as a primer for *in vitro* DNA replication[23].

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