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Abstract

Following a massive dose of CCl_4 in mice, a time study was made on the 2-hour incorporation of P^{32} into liver DNA and on the mitotic activity of liver cells. An increase in P^{32} incorporation began 30 hours after CCl_4, with a maximum at about 40 hours. Mitotic activity was not apparent until after DNA synthesis had reached its maximum. The radiation experiments (with varying time intervals between CCl_4, 800 r total-body x-ray, and sacrifice) showed that DNA synthesis was depressed when the mice were irradiated 0, 12, 72, or 96 hours after CCl_4, but that it was not affected when the radiation was given between 24 and 48 hours after CCl_4. Mitotic activity was absent 5 hours after radiation, began to return at 14 hours, and reached the control value at 26 hours. The results indicate that separate mechanisms are responsible for the effects of irradiation on DNA synthesis and on mitotic activity in liver.

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INTRODUCTION

The experiments to be reported were undertaken in order to study the timing of the synthesis of desoxyribose nucleic acid (DNA) with respect to mitosis and to determine whether sensitivity to irradiation during any one particular phase of the cell cycle could account for the gradual inhibition of DNA synthesis observed in earlier experiments.[1]

The ideal material for such a study would be a synchronously dividing cell population--which unfortunately is not available in mammalian tissue. The initial phase of liver regeneration therefore was chosen as an approximation to synchrony. Because it was expected that large numbers of mice would be necessary, regeneration was induced by carbon tetrachloride (CCl_4) rather than by partial hepatectomy.

Since the initial study by Brues et al.,[2] numerous papers have been written on the incorporation of precursors into DNA of livers regenerating after partial hepatectomy (e.g., References 3-8). A detailed analysis of histological and chemical changes in mouse livers following CCl_4 necrosis has been published.[9,10]

During the course of our investigation, irradiation studies on regenerating livers have been carried out in several other laboratories.[11-14]

Methods

Approximately 500 male A-strain mice weighing 22 to 27 grams were used. They were fed ad libitum. Liver necrosis was produced by the subcutaneous injection of 0.1 cc of a 35% solution of CCl_4 in sesame oil. Irradiation was carried out by an x-ray machine operated at 220 kv, 15 ma with added filtration of 1 mm Cu and 1 mm Al, and at a dose rate of approximately 15 r/min. During irradiation the mice were housed in a wooden box with nylon screen top and were free to move about.

At least 20 mice at a time (but usually a larger number) were injected with CCl_4, and half the animals were irradiated subsequently. A tracer dose of $Na_2HP^{32}O_4$ (20 to 25 μc) in isotonic saline was injected intraperitoneally at a given time after CCl_4 into the whole group, and they were sacrificed exactly 2 hours after the phosphate injection. In most of the experiments sections were taken for mitotic counts, and portions of several livers were pooled for inorganic phosphate specific activity determinations by the method of LePage.[15] The remaining tissue from two livers was pooled for the isolation of DNA and determination of its specific activity by a previously published method.[16]

Sections for mitotic counts were cut 6 μ thick and stained with H and E. In each liver, mitoses were counted in a minimum of 10 fields under high power (a total of approximately 700 parenchymal cells). Only cells in late prophase, metaphase, and anaphase were counted.

Results

Response to Carbon Tetrachloride

Figure 1 represents the DNA specific activities* that were obtained in livers of nonirradiated mice when P^{32} was administered at varying times after a massive dose of CCl_4. It can be seen from the graph that the rate of incorporation remained low until 30 hours after CCl_4, and then increased abruptly until a maximum was reached at about 40 hours. At this time the DNA specific activity was approximately 75 times the normal value for liver. It then declined gradually; however, even at 5 days the rate of incorporation was still six times normal. Throughout this interval the specific activity of the inorganic phosphate was 2.65 ± .12.

Figure 1 also indicates the mean mitotic counts in sections from the same livers as were used for the DNA measurements. Sections were scanned for mitoses in the nonparenchymal cells, but none were found. Significant mitotic activity in parenchymal cells was first observed at 44 hours, and the highest mean value was seen at 74 hours. Because there was great variability in the mitotic activity of livers examined at each time interval, the data have been summarized in Fig. 2 in the form of a histogram. From this

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* Throughout this paper specific activity is expressed as counts per minute per mg phosphorus divided by cpm injected per gram mouse. (To convert values in our previous publications to these units, multiply them by 25.)
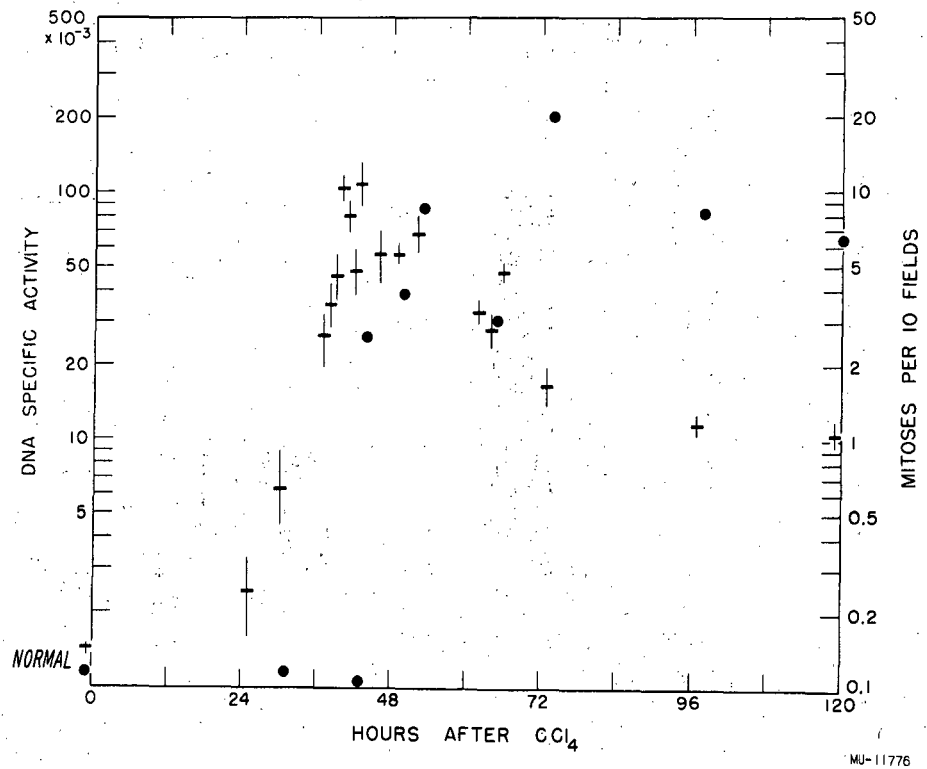
Fig. 1. Two-hour specific activities of liver DNA and mitotic counts after CCl₄.

Data were plotted on semilogarithmic scale because of wide range of values and not for theoretical reasons.

Values represent mean specific activities; vertical lines show standard error of the mean. Specific activities were calculated as counts per minute per milligram DNA phosphorus divided by cpm injected per gram mouse.

Mean number of parenchymal cell mitoses per 10 fields under high power.
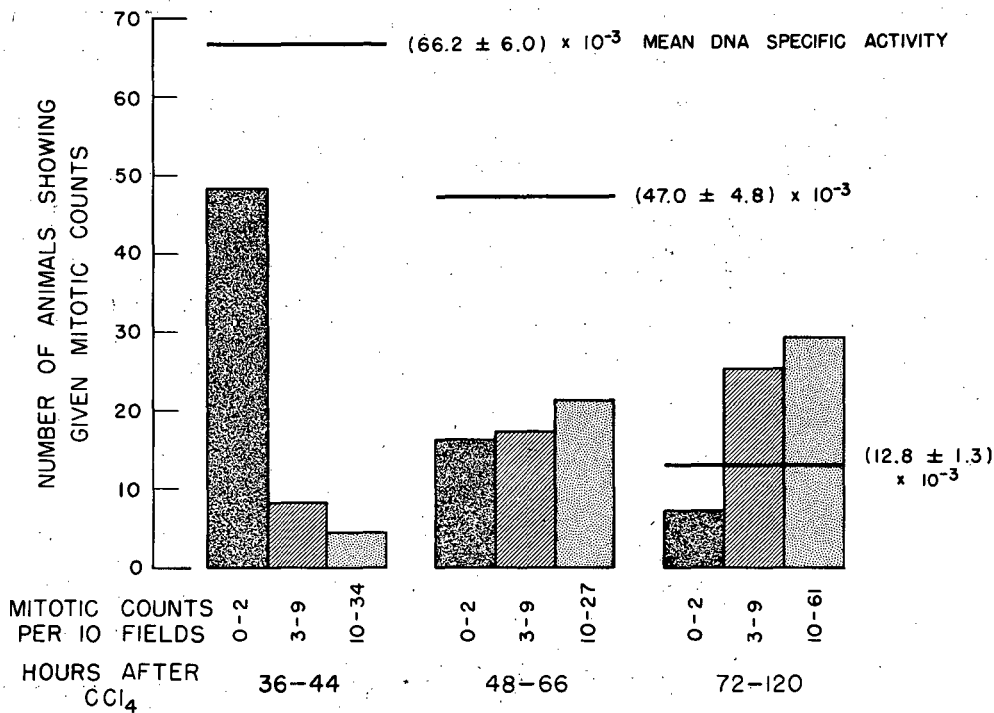
Fig. 2. Histogram summarizing mitotic counts at various time intervals after CCl_4.

it is evident that mitoses were rare during the 36- to 44-hour interval, at which time the DNA synthesis was at a maximum. From 48 to 66 hours an increasing number of livers showed mitotic activity while the rate of DNA synthesis was declining; and during the last time interval the mitotic activity was maximal while the DNA specific activities had dropped to approximately one-tenth of the peak value. Under the conditions of this experiment, the time between initiation of DNA synthesis and initiation of mitosis appears to be of the order of half a day.

Effects of Irradiation

The results of all the radiation experiments are summarized in Table I. They are expressed as the ratios of the liver DNA specific activities of irradiated to unirradiated mice and were calculated separately for each experiment. (The specific activity of inorganic phosphate for all the irradiation experiments was $2.49 \pm .14$, which is not significantly different from the control value.)

When normal mice are irradiated with 800 r, the maximum depression in DNA synthesis does not occur until one day postirradiation.[1] Therefore the initial experiments in this series were carried out with a 24-hour interval between irradiation and the injection of P^{32}. When the mice were irradiated at 0, 12, 72, or 96 hours after CCl_4, the expected reduction in DNA synthesis was found. However, irradiation at 24 or 41 hours after CCl_4 produced no effect on the DNA specific activity when measured 24 hours later. It seemed possible that this lack of effect could be due to rapid recovery after radiation rather than to radioresistance. To test this possibility, a number of experiments were carried out with shorter time intervals between irradiation and the injection of P^{32}, but no significant depression in incorporation was found, even when the radiation dose was increased to 2000 r. Owing to the great variability, in both the control and irradiated groups, the standard errors in some experiments were very large, and a small depression in specific activity would not be evident. When all experiments involving irradiation between 24 and 48 hours (the resistant period) were combined, the DNA specific activities were as follows:

for the 800 r series:

   controls $(57.6 \pm 5.2) \times 10^{-3}$,
   irradiated $(55.0 \pm 4.8) \times 10^{-3}$;

for the 2000 r series:

   controls $(52.2 \pm 6.3) \times 10^{-3}$,
   irradiation $(51.7 \pm 10.1) \times 10^{-3}$,

confirming the existence of a radioresistant period.

Table I lists two experiments in which the DNA specific activities of the irradiated livers were significantly higher than the controls. In one of these (irradiation at 12 hours and P^{32} 27 hours later), the mean DNA specific activity in the irradiated group was $(234 \pm 21) \times 10^{-3}$, which was twice the highest control value observed in any experiment.

Table I

Effect of 800 r on DNA specific activity at various times after CCl_4

Time CCl_4 to x-ray (hr)	Time x-ray to P^{32} (hr)	DNA specific activity (ratio of irradiated to control) and standard error of the ratio
0	24	.20 ± .08
12	17	.15 ± .05
12	24	.37 ± .13
12	27	2.08 ± .40
24	16	.90 ± .37
24	24	.97 ± .19
24	21	.98 ± .70[a]
34	3	.93 ± .28
34	3	.82 ± .26[a]
36	5	1.07 ± .41
41	24	.98 ± .12
48	3	1.03 ± .29
48	3	1.08 ± .17[a]
60	1	.86 ± .12
60	3	1.52 ± .22
72	24	.45 ± .09
72	24	.69 ± .17
84	12	.31 ± .12
96	24	.39 ± .08

[a]   mice received 2000 r

In some of the experiments mitoses were counted in sections of each liver used for the measurement of DNA specific activity. The mean mitotic counts in the control and irradiated livers, together with the ratios of irradiated to control DNA specific activities for each experiment, are listed in Table II. The data are arranged according to increasing time between irradiation and sacrifice, and demonstrate that mitoses were absent 5 hours after radiation, were beginning to return at 14 hours, and were again normal in frequency at 26 hours. All mitotic figures were included in the count, and no attempt was made to score the number that were abnormal. The experiments demonstrate clearly that after irradiation mitoses may be absent while the incorporation into DNA is unaffected, and, conversely, they may occur at a normal rate at a time when DNA synthesis is depressed.

In many experiments the incorporation of P^{32} into DNA of spleen and small intestine was also measured. CCl_4 treatment had no significant effect on the DNA specific activities of these tissues. Irradiation in all cases produced a depression quite analogous to that found earlier in untreated mice,[1] indicating that the radioresistance found in livers after CCl_4 is not a general phenomenon.

Discussion

Figure 1 demonstrates a very striking increase in the rate of DNA synthesis beginning about 30 hours after a necrotizing dose of CCl_4. The timing and the maximum specific activity observed are in general agreement with the results of Jardetzky et al.,[11] who studied mouse livers after partial hepatectomy. This indicates that whether regeneration is induced by CCl_4 or partial hepatectomy, the timing is similar. As noted by Yokoyama et al.[17] regeneration apparently takes place later in the mouse than in the rat, where maximum rates of DNA synthesis have generally been reported to occur at 20 to 24 hours. It is of interest that such a sharp peak in DNA specific activity can be observed after CCl_4 administration in spite of the fact that the degeneration and regeneration of liver cells overlap.[10]

The initiation of mitotic activity on the second day, with maximum activity on the third day, is in agreement with Wilson et al.[18] Figures 1 and 2 demonstrate clearly that the maximum rate of DNA synthesis precedes active mitosis. The data suggest a time interval of the order of half a day between initiation of the two processes. This is in keeping with the current evidence, recently reviewed by Swift,[19] that DNA synthesis occurs during interphase. Since there was great variability, both in the DNA specific activity and in the mitotic activity, the timing of the initiation of the two processes is only an approximation. Furthermore, the mitotic counts include only parenchymal cells, whereas the DNA was necessarily derived from all cell types in the liver. Considering all the possible sources of variation, the ratios of the maximum regeneration values to normal values agree fairly well for DNA specific activity (75) and mitotic activity (180).

Table II

Mitotic activity and DNA specific activity after irradiation

Time x-ray to sacrifice (hr)	Mitoses per 10 fields		DNA specific activity (ratio of irradiated to control)
	Control	Irradiated	
5	5.7	0	1.03
5	11.5	0	1.08
14	9.6	2.0	0.31
26	6.3	6.4	0.39
26	5.9	6.6	0.45
26	3.9	6.5	0.97

The irradiation experiments are very difficult to interpret. They were originally undertaken because of the results of Howard and Pelc,[20] who found in bean root cells that irradiation during interphase caused a delay in DNA synthesis if the irradiation occurred before synthesis, but had no effect once synthesis had begun. In some respects our results (Table I) could be fitted into a similar scheme. If the mice were irradiated between 0 and 12 hours after CCl_4, a depression in DNA specific activity was observed, whereas irradiation between 24 and 48 hours had no effect. However, a number of observations suggest that some other explanation will be found for the pattern of irradiation sensitivity and resistance in liver.

Although for mice irradiated at 12 hours the specific activities measured 17 or 24 hours later were lower than controls, they were double the control values if they were measured 27 hours later. This suggests that 800 r merely delayed the onset of DNA synthesis slightly, and perhaps the very high incorporation was due to somewhat improved synchronization.

A period of radioresistance in mouse livers regenerating after CCl_4 seems to be well established by our experiments. Jardetzky et al.,[11] on the other hand, found an immediate depression in DNA specific activity when mouse livers regenerating after partial hepatectomy were irradiated with 2000 r. Using regenerating rat livers, Thomson et al.[13] observed a depression in DNA synthesis at various times after 800 r, while Holmes and Mee[12] found a resistant period analogous to that in our experiment after 450 r but an immediate depression after 2200 r. CCl_4 is thought to produce severe anoxia in the liver,[21] and it is possible that this contributes to the radioresistance in our mice.

It should be pointed out that the induction by radiation of a direct biochemical lesion in DNA synthesis has been questioned recently.[1,22] The inhibition of DNA synthesis in liver may be due, in part at least, to abscopal effects, such as the massive release of adrenal hormones or the reduction in food intake. Recent experiments have demonstrated that the incorporation of P^{32} into normal liver DNA is reduced to about 40% by a 24-hour fast.[23] When mice were fasted for one day beginning 72 hours after the administration of CCl_4, the depression in DNA specific activity was identical to that observed after 800 r. Reduced food intake, however, could not account for the immediate depression in DNA synthesis observed after higher radiation doses.

Table II demonstrates that there can be a complete mitotic inhibition at a time when there is no measurable inhibition of DNA synthesis. A similar finding has been reported by Holmes and Mee[12] for regenerating rat livers, and by this laboratory for Ehrlich ascites cells.[24] Howard and Pelc[20] were the first to point out that the radiation inhibition of mitosis must be due to a process that is independent of any effect on DNA synthesis.

The existence of a separate mechanism for the initiation of mitosis (as illustrated by the radiation experiments) implies that the time interval between DNA synthesis and mitosis may depend on the particular physiological conditions of the cells at the time of measurement. The interval observed in our experiment might, for example, be longer than that after partial hepatectomy if CCl_4 poisoning created an unfavorable environment for the initiation of mitosis.

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