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EFFECTS OF FLUORODEOXYURIDINE ON DNA REPLICATION, CHROMOSOME BREAKAGE, AND REUNION*

BY J. HERBERT TAYLOR, WILLIAM F. HAUT, AND JEANNE TUNG

DEPARTMENT OF BOTANY AND DEPARTMENT OF ZOOLOGY, COLUMBIA UNIVERSITY

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Fluorodeoxyuridine (5-fluorouracil deoxyriboside, FUDR) is phosphorylated in some cells. The fluorodeoxyuridylate formed is a highly active inhibitor of the enzyme, thymidylate synthetase;¹ therefore, the conversion of deoxyuridylate to thymidylate is blocked. Since this is the only pathway for the synthesis of thymidylate in most cells and their pool of thymidylate is usually very small, DNA replication is quickly blocked. The block can be overcome by supplying thymidine from an exogenous source. Since thymidylate functions primarily as a precursor

in DNA synthesis, the inhibitor is restricted in its effects on cellular metabolism. However, some side effects may be produced by conversion of FUDR to fluoro-uridylylate which is known to be incorporated into RNA². These side effects can be reduced by supplying cells with an excess of uridine which apparently does not compete with FUDR in its original phosphorylation.

Although FUDR stops DNA replication quickly, cell division continues for several hours in root cells of *Vicia faba*. Most of the cells which have completed their synthesis of DNA and are in G₂ (the gap of about 4 to 6 hr in the cycle between the end of synthesis and division) should in the absence of side effects continue through the division unless thymidylylate is required in some process other than chromosome reproduction. During the first three hours following the immersion of roots in FUDR, the cells arriving at division appear quite normal. By the fourth hour, gaps appear in some chromosomes at anaphase and a few free fragments are produced. By the sixth hour, only a few cells are still reaching division and many of these show a remarkable shattering of the chromosomes. However, if thymidine is supplied to the cells within about an hour before they divide, most or all of the lesions are healed and the chromosomes appear quite normal when they reach anaphase. The experiments reported here also indicate that not only these lesions but also breaks produced by radiation require thymidylylate for repair and reunion of ends produced by breakage. Based on these results, a general hypothesis for the reunion of chromosomes is proposed which should aid in our understanding of genetic recombination.

Material and Methods.—Seedlings of *Vicia faba* were grown in glass vials with roots immersed in a nutrient mineral solution to which was added the various chemicals as desired. Secondary roots about one centimeter long and growing in the dark at 19–20°C were used for the experiments except where other conditions are indicated in the text. Roots were fixed in alcohol-acetic acid (3:1) usually for 1–3 hours, hydrolyzed in 1 N HCl for 6–7 min., stained by the Feulgen reaction, and squashed on slides under cover glasses in 45% acetic acid. After freezing on dry ice, the cover glasses were removed while the preparations were frozen and the cells dehydrated by immediate immersion in absolute ethanol. Preparations were made permanent by mounting in Diaphane or Euparal.

Seedlings varied enough in their mitotic index so that data were collected by sampling 2–3 secondary roots of a single seedling at successive intervals when possible. After examining many roots, 200–350 anaphases per root (terminal 2 mm) was found to be typical of healthy roots growing under our conditions at 19–20°C. When seedlings deviated much from this range, they were not used for experiments.

X-radiation was supplied to unshielded roots by a General Electric Medical D-3 Unit operating at 75 Kvp and 10 milliamperes. Roots were irradiated in air at about 25°C. With only the inherent filtration (equivalent to 1 mm. of aluminum), the unit delivers 25 r in about 18 sec to roots placed 15.75 inches from the target.

Results.—When roots were immersed in concentrations of FUDR varying from 10⁻⁴ M to 10⁻⁸ M, effects on the mitotic index were observable within a few hours. Figure 1 shows the change in number of divisions per root at various times after immersion in the inhibitor. At concentrations of 10⁻⁶ M or greater, an effect on the mitotic index is evident within 2 hours, but at 10⁻⁷ M concentration, the effect

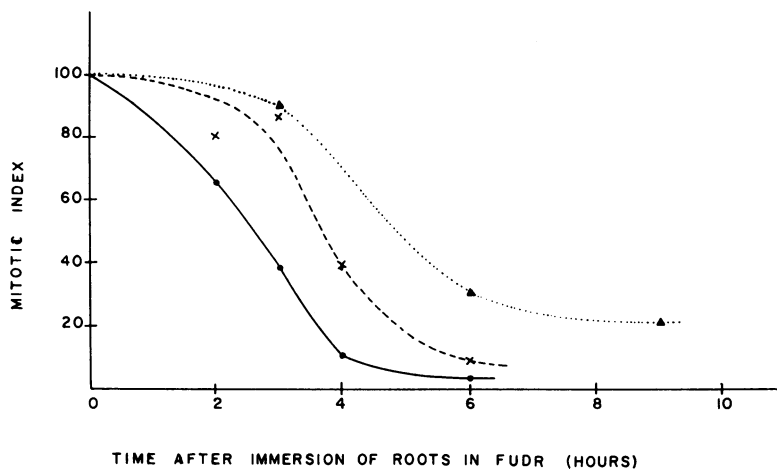


FIG. 1.—Graph showing the effects of FUDR on the mitotic index. The lower curve shows the effects of 10^{-6} M FUDR; the middle curve shows the effects of 10^{-6} M FUDR in combination with 10^{-7} M uridine and the upper curve shows the effects of 10^{-7} M FUDR.

is small during the first 3 or 4 hours. Uridine given simultaneously with the FUDR affords some protection. No data are presented for the higher concentration, but counts of anaphases showed that the mitotic rate dropped a little faster at 10^{-5} and 10^{-4} M than at 10^{-6} M. At 10^{-8} M, there was some decrease, but the effects of concentrations lower than 10^{-7} M are not of interest in this report. Thymidine and BUDR (bromouracil deoxyriboside) reduce the effects of FUDR when given simultaneously. When thymidine at a concentration of 10^{-3} M is used with 10^{-6} M FUDR, the mitotic index drops less than 50 per cent during a 12-hr period, but when used with 10^{-5} M FUDR, the drop in frequency of divisions is similar to that obtained with 10^{-7} M FUDR used alone.

At all of these concentrations, most of the cells which reached anaphase during the first 3 hours at 20°C have intact chromosomes (Table 1). In the early experi-

TABLE 1
FREE FRAGMENTS AT ANAPHASE AFTER TREATMENTS WITH FUDR

Concentration of FUDR	Ratio of Anaphases with Fragments to Total Anaphases after Various Times in FUDR						
	0 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr
10^{-7} M*	0/63	1/61		3/83	0/50	3/18	1/21
10^{-6} M*		8/76		17/35	11/11		
10^{-6} M and 10^{-4} M uridine	0/82	0/76	4/90	35/75	8/12		
10^{-6} M	0/71	0/83	13/46	6/8	14/17		
10^{-6} M and 10^{-4} M uridine (2 hr); then 10^{-4} M thymidine and 10^{-4} M uridine		0/138	1/47	0/23	0/32		
10^{-6} M* (2 hr); then 10^{-4} M thymidine and 10^{-4} M uridine		4/77	2/63	0/27	0/8		
5×10^{-5} M*		1/74		19/56	7/7		

* Treated at room temperature $23-24^{\circ}\text{C}$; all others were grown and treated at $19-20^{\circ}\text{C}$.

ments, which were carried out at room temperature ($23-25^{\circ}\text{C}$), a few free fragments began to appear within 2 hours at the higher concentrations. After 3 hours at 20°C in 10^{-6} M FUDR, some cells begin to show breaks, and by 4 and 6 hours, many of the anaphases as well as late prophase and metaphases showed a remark-

able shattering of the chromosome complement (Fig. 2). Sometimes, only the centromeres with short pieces of chromatin attached reached the poles. The remainder of the chromosome arms were left scattered near the center of the spindle.

At a concentration of 10^{-7} M FUDR, essentially no breaks appeared in cells that reached division during the first 6 hours in spite of the fact that this concentration during the same period results in a marked drop in mitotic index. On the other hand, 10^{-6} M FUDR produced nearly a maximum effect on fragmentation. Higher concentrations, 10^{-5} or 10^{-4} M, produced a quicker effect on the mitotic



FIG. 2.—(a) Anaphase of *Vicia faba* showing gaps and breakage 4 hours after immersion in 10^{-6} M FUDR. (b) Anaphase from a root treated 6 hours in a solution of 10^{-6} M FUDR.

index, but cells which were able to reach division showed damage similar to that produced by 10^{-6} M FUDR. The indications are that the chromosomal lesions are due to interruptions in DNA replication in cells which are just finishing the phase of synthesis when affected by the analog. Most of the cells in the DNA synthetic phase stop their progress toward anaphase and, as later experiments showed, wait until thymidylate is available. After a short lag period, they come to division with chromosomes intact. However, a fraction of the cells that are near the end of the stage of DNA replication have no mechanism to halt the progress toward division. They reach division with gaps in their chromosomes. Evidently, only one of the two subunits of DNA is missing at any one locus, for previous experiments have shown that one subunit is new and one is the original DNA.³ If we assume that the chromatid is a single DNA double helix, as much of the present evidence indicates,³ the chromosomal lesions consist of regions in which the DNA exists as single chains. The characteristic folding and/or coiling may be interrupted at these loci. In any case, the lesions appear as gaps (stretched regions). Presumably, the torsions and tensions, produced by chromosomal coiling at prophase and the anaphase movement, often result in complete fragmentation. The breaks are somewhat localized to the regions which replicate late in *Vicia*, but detailed studies on the sites of breakage have not been attempted yet. The nucleolus organizer region (NOR) often breaks first. However, in collecting the data reported in Tables 1 and 2, breaks at these four loci, two in each anaphase

TABLE 2

EFFECTS OF FUDR ON REUNION OF CHROMOSOMES BROKEN BY X-RADIATION
(Ratio of aberrant anaphases to normal anaphases in cells fixed 4 hours after 25 r)

	Control	10^{-6} M FUDR, 10^{-4} M uridine (15 min before X-raying)	10^{-7} M FUDR (30 min before X-raying)	10^{-7} M FUDR, 10^{-6} M uridine 30 min before X-raying
Chromatid bridge and fragment	35/546	1/160	1/86	2/179
Free fragments (1-3)*	90/546	134/160	12/86	64/179
Gaps only	141/546	19/160	23/86	52/179
Per cent of anaphases with a chromatid bridge and fragment				
	Control	6.4%		
	10^{-6} M FUDR	0.6%		
	10^{-7} M FUDR	1.1%		

* Except cells treated with 10^{-6} M FUDR, some of which had more than three fragments per cell.

group, have not been included. In the squashing technique, portions of chromatids distal to the NOR are displaced in control material frequently enough to make scoring of lesions at these loci very difficult and somewhat unreliable. Other points of frequent breakage, or the production of gaps, in the earliest anaphases to show lesions often appear in the positions reported by Woodard and Swift⁴ to be late in reproduction, i.e., in the long arms of several of the 10 short chromosomes at loci proximal to the centromere and near the mid-region in some. However, *Vicia* is not very satisfactory for detailed study of the correlation between the lesions and late reproducing regions, because some incorporation (replication) occurs along most of the length of the chromosomes even when tritiated thymidine is available only at the end of the synthetic period.

The threshold effect, i.e., the transition from no breakage at a concentration of 10^{-7} M to nearly a maximum effect at 10^{-6} M indicates that the enzyme, thy-

midylate synthetase, must be blocked quickly to produce the lesions. The slower action at 10^{-7} *M* concentration probably allows the cells, which cannot stop their progress to division, to obtain enough thymidylate to finish their chromosomes.

A remarkable feature of the fragmentation is the failure of any of the broken chromatids to reunite. In the hundreds of anaphases and metaphases examined, not a single reunion was seen. If the lesions are due to a failure of DNA replication because of the lack of thymidylate, supplying the cells with thymidine after the lesions were produced might result in their healing. This proved to be true (Table 1).

Thymidine at a concentration of 10^{-4} *M* resulted in essentially complete healing of the lesions when given two or even 3 hours after the FUDR. Even in these cells, no evidence of chromatid exchanges or reunions of any kind were seen. This could mean that the lesions are really potential breaks of chromatids, i.e., interruptions in only one subunit. If the synthesis is completed before the pieces are dislocated in division, no exchanges occur.

However, these observations suggested experiments to test the reunion of chromosomes broken by radiation in the absence of thymidylate. The experiment was feasible because treatment with 10^{-7} *M* FUDR stops division and presumably DNA replication without producing any visible breaks in 6 hours at 20° C. After several trials, a dose of 25 r was selected as the most suitable. This dose produced many chromatid fragments and gaps in cells arriving at division 4 hours later and also resulted in a few chromatid bridges, accompanied by a single free fragment. These bridges, which indicate reunion of chromatids, can be clearly distinguished from the sub-chromatid or sticky bridges often seen in the early hours following radiation.

In roots X-rayed without treatment in FUDR, the dose of 25 r produced 6.4 per cent of the anaphases with chromatid bridges in cells arriving at division 4 hours later (Table 2). When roots were pretreated 30 min with 10^{-7} *M* FUDR before X-raying and grown in the presence of the inhibitor until fixation 4 hours later only 1.1 per cent of the anaphases had chromatid bridges, while the number of fragments and gaps was not very different from the control (Table 2). When the concentration was raised to 10^{-6} *M*, the frequency of bridges apparently dropped, 0.6 per cent, even though there were many more breaks. The scoring of gaps and fragments is a difficult task at best, and when these are so numerous, the numbers counted may be taken only as an approximate measure of the radiation damage. However, the data on chromatid bridges are clear enough to make it certain that relatively little reunion of a type that will withstand the stretching of anaphase can occur without thymidylate. Probably none occurs in the complete absence of thymidylate, for there are indications that some is available for an interval after immersion of roots in FUDR (see the previous experiments on the failure of 10^{-7} *M* FUDR to produce lesions).

The next experiments were carried out to test the effect of thymidine and BUDR on the reunion of chromatids in the presence of the block by FUDR (Table 3). Both substances allowed reunion at nearly the level of the control. The number of free fragments and gaps are comparable to those in the control group. These results appear to confirm the notion that thymidylate is required for the stable reunion of broken chromatids.

Other base analogs with substitutions at the 5-position produced essentially no

TABLE 3

REVERSAL OF THE EFFECTS OF FUDR ON REUNION OF CHROMOSOMES BROKEN BY X-RADIATION
(Ratio of aberrant anaphases to normal anaphases in cells fixed 4 hours after 25 r)

	Treatment: Roots immersed in 10^{-7} M FUDR and 10^{-3} M uridine 30 min before X-raying; 10^{-4} M thymidine or 10^{-4} M BUDR added after X-radiation	
	10^{-4} M thymidine	10^{-4} M BUDR
Chromatid bridges and fragments	21/384	9/162
Fragments (1-3)	43/384	31/162
Gaps only	65/384	32/162
Per cent of anaphases with a chromatid bridge and fragment		
Control	6.4% (see Table 1)	
10^{-4} M thymidine	5.4%	
10^{-4} M BUDR	5.5%	

effects on the chromosomes and little if any effect on the mitotic index over a period of 12-24 hours. 5-amino uracil, 5-hydroxyuridine, BUDR, thymidine, and deoxyuridine in concentrations as high as 10^{-3} M produced no measurable cytological effects. Some of these may have produced more stretching and some breakage in the nucleolus organizer regions, but, as mentioned earlier, untreated cells vary considerably in the amount of displacement of the pieces distal to the NOR. This makes quantitative evaluation of these effects very difficult. 6-azauridine, which is a specific inhibitor of orotidylic acid-decarboxylase,⁵ was also ineffective, perhaps because the cotyledons of the bean seedlings provide a reserve of pyrimidines for the root.

Discussion.—The results reported above support the idea that at least part of the linear axis of the chromosome is DNA. The failure of its replication to be completed results in lesions at many sites which can lead to complete breakage. The question may be asked whether the lesions result from the absence of single nucleotides (thymidylate) in the DNA chains or long stretches of single-stranded DNA. While the evidence is insufficient to provide a definite answer, the cytologically visible gaps suggest very long stretched regions at the molecular level. In addition, the failure of broken chromatids to rejoin in the absence of thymidylate suggests that polymer synthesis which requires all four nucleotides may be involved in both the rejoining and repair mechanisms.

The enzyme, DNA polymerase, studied by Kornberg and associates,⁶ adds 5'-nucleoside triphosphates at the 3'-hydroxyl group on the end of chains. The chains would be unable to grow from the opposite end by the action of this enzyme. The results with the FUDR suggest the hypothesis that this enzymatic mechanism operates in the repair of DNA with broken chains.

A broken chain would be able to grow from one end only and it would presumably copy the antiparallel chain as it grows. The displaced polynucleotide chain might be cast off, perhaps as single-stranded DNA. In the rejoining of two broken pieces of DNA (Fig. 3), two such fragments would be cast off. The assumption is made, although it is perhaps not a necessary one, that the copying continues to the end of a replicating unit of which one may suppose there are many in a large chromosome. In random rejoining, 50 per cent of the contacts might be supposed to occur between nongrowing ends. Such contacts could lead to nonunion, but the growth of the two short chains with growing ends might bypass the original contact to copy each other or, alternatively, lead to a second opening of the break.

In the repair of the lesions produced by FUDR, according to this concept, no

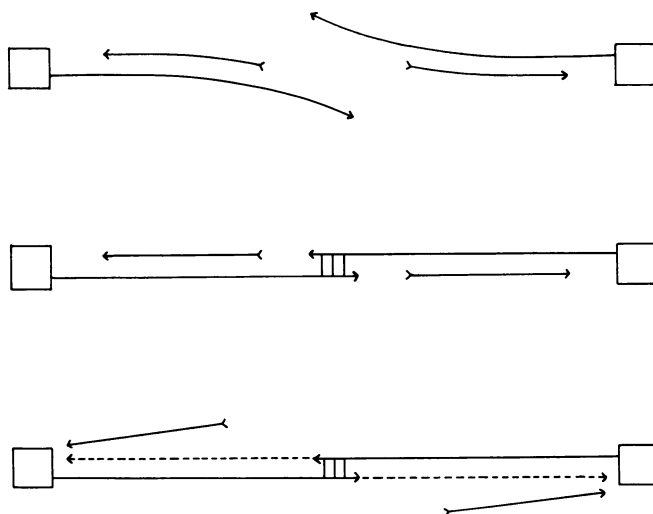


FIG. 3.—Diagram of the steps involved in the reunion of broken chromosomes according to the hypothesis proposed in the text. Fragments of two replicating units of broken chromosomes are shown. The lines represent sub-units of a chromatid, presumably single polynucleotide chains.

fragment would be eliminated, but growth of chains would continue from the sites at which it ceased in the absence of thymidylate.

This hypothesis of repair and rejoining of DNA chains is attractive for explaining not only the results reported here but also many of the reports which are accumulating on genetic recombination, especially the aberrant segregation in ascomycetes reported by Olive,⁷ Case and Giles,⁸ and Rizet⁹ and the formation and segregation of the DNA heterozygotes in phage.¹⁰ The proposed mechanism would result in polymer synthesis (DNA chains) at any time a break occurred which could be repaired. Since there is evidence that breaks in chromosomes can rejoin at periods in the cell cycle outside the principal DNA synthetic phase, the hypothesis implies that DNA replicating units (or parts of these) can replicate more than once in each division cycle if a break is produced in one or both polynucleotide chains. Such double replication and the casting off of chains in limited regions of the genetic map provide a mechanism to explain aberrant segregation. Reciprocal exchanges and/or miscopying could occur as part of this repair when homologous DNA helices were paired or close together, and furthermore, recombination in higher forms would not necessarily be limited to the principal DNA synthetic interval. It could occur before, during, and after this stage if homologous DNA helices were close enough together to rejoin with each other when broken.

Several observations may be mentioned which tend to support or could be explained by the proposed hypothesis. In cell-free systems, the treatment of primer DNA in ways that would break and expose growing ends appears to promote polymer synthesis.¹¹ Das and Alfert¹² also reported a temporary increase of the number of cells synthesizing DNA and an increase of the rate in some cells following a moderate dose of irradiation. Another anomalous case reported by Baeckeland *et al.*,¹³ in which treatment of mammalian cells in culture with an acid deoxy-

ribonuclease led to the accumulation of DNA in the cytoplasm, might be explained by this repair mechanism. If the enzyme broke chains and provoked the elimination of fragments of DNA, these could accumulate in the cytoplasm in detectable amounts.

Summary.—Growth of roots of *Vicia faba* immersed in solution of fluorodeoxyuridine, a specific inhibitor of the enzyme thymidylate synthetase, results in chromosomal lesions which can lead to extensive fragmentation during cell division. At concentrations of 10^{-7} and 10^{-6} M, the frequency of mitotic figures is reduced after a few hours. Most of the reduction occurs as would be predicted if those cells that had finished DNA synthesis and those close to finishing divided once in the presence of the inhibitor. Other cells at earlier stages are blocked in their progress toward division. The last cells to reach division show a striking cytological damage. Up to 3 hours, the anaphases have chromosomes that are normal in appearance. After this time, gaps appear along with a few fragments. The damage becomes progressively greater in the last chromosomes to reach division. The lesions heal if either thymidine or BUDR is supplied to the cell at least an hour before they reach anaphase. Since 10^{-7} M FUDR does not produce visible lesions within 6 hours but does apparently stop DNA synthesis within a short interval, the effects of thymidylate deficiency on the reunion of chromosomes broken by X rays could be studied. The experiments indicate that reunion requires DNA synthesis. Immersing the roots in solutions of thymidine or BUDR after irradiation in the FUDR-blocked condition allows reunion almost to the same extent as in the controls.

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