THE FREQUENCY OF SISTER CHROMATID EXCHANGES FOLLOWING EXPOSURE TO VARYING DOSES OF H^3-THYMIDINE OR X-RAYS

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ABSTRACT

In the Chinese hamster cell line CHEF-125, sister chromatid exchanges occurred at a rate of a little higher than one per three chromosomes for each cell cycle. The exchanges were detectable by labeling with H^3-thymidine and autoradiographic analyses of chromosomes at the second and subsequent metaphases after labeling had occurred. To test the hypothesis that sister chromatid exchanges are caused by radiation, cells were incubated in media with different amounts of H^3-thymidine. No statistically significant change in the exchange rate was detected over 100-fold range of variation in the amount of incorporated H^3-thymidine (determined by grain counts of autoradiographs). We have concluded that sister chromatid exchanges are not caused by tritium radiation and therefore are spontaneous events. Cultures were also irradiated with acute doses of x-rays up to 200 r and scored for sister chromatid exchanges. Between zero and 50 r there was a statistically significant increase in the rate of exchanges. This is interpreted as evidence that x-rays can induce some exchanges, although the majority of these events are probably spontaneous.

INTRODUCTION

Sister chromatid exchanges were first observed by Taylor and collaborators (14–16) in *Vicia* and *Bellevalia* metaphase chromosomes labeled with H^3-thymidine. At the second metaphase after exposure to the tritiated nucleoside, usually only one of the two sister chromatids of each chromosome was labeled; when both chromatids contained label, the labeled segment(s) on one chromatid was matched by an unlabeled segment(s) on the sister. This variation in the segregation pattern of labeled DNA in chromosomes was interpreted by Taylor, on the basis of a two-stranded model for premitotic chromatids, as due to breaks involving both strands of each of two sister chromatids, followed by reunion restricted by a difference in the two strands of each chromatid. Prescott and Bender (11) recently confirmed Taylor's findings (14, 15) of semiconservative segregation of DNA and sister chromatid exchanges in metaphase chromosomes of mammalian cells grown *in vitro*. In both laboratories, however, the label used was tritium, and it has been suggested by Plaut (9), from theoretical calculations, that the β-decay of tritium might deliver doses of radiation high enough to cause extensive chromatid breakage. Taylor (15) considered the possibility that sister chromatid exchanges might be induced by the

radiation from incorporated tritium, but his later experiments failed to support this thesis (14).

Several reports of chromosome breakage induced by H^3-thymidine incorporation have appeared in the last few years (1, 7, 8, 17); generally, chromosome damage could be detected after exposure to levels of H^3-thymidine as low as those used in which sister chromatid exchanges were observed. If sister chromatid exchanges were also radiation-induced, their frequency might be expected to vary closely with the corresponding frequency of conventional chromatid aberrations, since such exchanges might represent a class of restituted isochromatid breaks made visible by the differential labeling of the two sister chromatids. If this were so, sister chromatid exchanges might conceivably be a useful, sensitive measure of induced chromosome breakage.

Since a dose-dependence would be expected if exchanges were radiation-induced, we compared the frequency of exchanges in cells exposed to different doses of H^3-thymidine added to the nutrient medium and in cells exposed to different doses of x-rays (also necessarily labeled with H^3-thymidine). The term "exchanges" in this paper refers to sister chromatid exchanges only, unless otherwise specifically indicated. We used Chinese hamster strain CHEF-125 because of the extensive background knowledge on the chromosome number, generation time, x-ray-induced chromatid aberrations (2), and pattern of H^3-thymidine labeling of these cells (11).

MATERIALS AND METHODS

Cell Line

The Chinese hamster cell strain CHEF-125, derived from Yerganian's line 1290-2 was used (11). This strain has a modal number of 22 chromosomes, with distribution around this value. The cells were cultured in Puck's fibroblast medium (12), supplemented with 15 per cent fetal calf serum; 0.25 per cent 1:250 trypsin was rountinely used for subculture.

Thymidine Labeling

Cells plated in 55-mm petri dishes were incubated for 1 or 2 hours in medium containing various amounts of H^3-thymidine (Schwarz BioResearch, Inc., Orangeburg, New York). Starting with a concentration of 2.7 μc/ml (1.2 c/mM) in the medium, serial dilutions were made with growth medium containing 0.14γ/ml of unlabeled thymidine, down to a concentration of 0.05 μc/ml. The cultures were therefore exposed to a constant total concentration of thymidine, while the specific acitivty was varied from 1.2 to 0.02 c/mM. Under these conditions of exposure the uptake of label varies linearly with the total activity in the medium (6). At the end of the labeling period the cultures were washed with balanced salt solution (BSS) and reincubated with medium containing unlabeled thymidine at a concentration ten times higher than in the labeling medium.

X-Irradiation

A Phillips constant potential x-ray machine was used, operated at 250 kv and 15 ma. One mm of Al and 1 mm of Cu were added to the inherent filtration, giving a total hvl of 2 mm of Cu. The TSD was 50 cm; the exposure rate, measured in air with a Victoreen dosimeter, was about 50 r/minute. The cultures were irradiated in 55-mm petri dishes, with 3 ml of growth medium.

Chromosome Preparations

Colchicine was added to the cultures (final concentration, 10^{-7} M) about 10 hours before fixation. After treatment with hypotonic saline (20 per cent BSS) for a few minutes, metaphases were harvested and fixed in 5 per cent neutral formalin (30 to 60 seconds), and the chromosomes isolated by adding an equal amount of ethyl alcohol-acetic acid fixative (3:1), and air-dried on microscopic slides. This method, described elsewhere (10), yields well spread chromosome complements, free of cytoplasm.

Autoradiography

The slides were dipped in NTB-2 liquid emulsion (Kodak) at 44 to 46°C, air-dried, and exposed for different lengths of time, up to 108 days. They were developed for 2 minutes in D-11 developer at 20 to 22°C, fixed for 3 minutes, washed in running water for 20 minutes, stained with 0.25 per cent aqueous toluidine blue at pH 6, and mounted with Euparal.

RESULTS

Three series of petri dishes were plated with 10^5, 5×10^4, or 2.5×10^4 cells per dish. Thirty hours later the cultures were exposed for 2 hours to different levels of H^3-thymidine in the medium, ranging from 0.05 to 2.7 μc/ml (see Materials and Methods). Since the generation time of strain CHEF-125 in our culture conditions is approximately 17 hours, a mixture of second and third division metaphases is to be expected between 18 and 36 hours after exposure. However, because incorporation of high levels of H^3-thymidine was suspected of causing some mitotic delay, colchicine was not added to the first series of cultures (10^5

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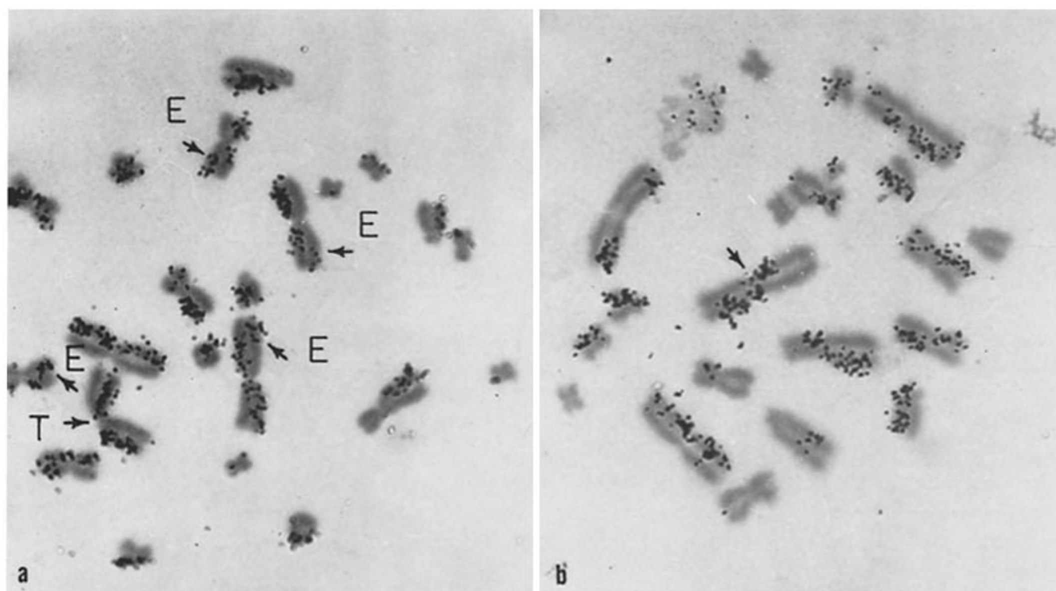
cells) until 27 hoursafter exposure to H^3-thymidine, and metaphases were harvested 8 to 12 hours later. The second (5×10^4 cells) and third (2.5×10^4 cells) series of dishes were colchicine-treated and harvested 8 to 25 hours after the first series.

Fig. 1 shows patterns of DNA labeling in chromosomes at the second and third metaphases after H^3-thymidine incubation. Interpretation of the segregation patterns is given in previous papers (11, 14, 16). Each chromosome set contains a number of sister chromatid exchanges. Table I shows that the first series of cultures gave a mixture of second, third, and fourth division metaphases, while second-division metaphases were markedly reduced in the second series, and almost absent in the third. It is clear from Table I ("Total" column) that the total number of labeled metaphases, at all doses, decreased with time and, furthermore (last column), that the percentage of first plus second metaphases at 37 hours at least increased with dose. These results are evidence of a selective advantage for unlabeled cells. It seems clear that either cell death or dose-dependent division delay is responsible for the results in Table I.

Table II and Figs. 2 *a* and *b* show the total number of sister chromatid exchanges observed in second and third division metaphases as a function of dose. In second division metaphases, any unlabeled region following a labeled segment of a chromatid and matched by the opposite pattern in its sister chromatid was scored as one exchange. In third division metaphases, exchanges were scored as belonging to the second or third cycle after labeling, according to whether the sister chromatid showed the opposite pattern or was totally unlabeled (Fig. 1). Often the label appeared to switch from one chromatid to the other at the level of the kinetochore. This type of configuration was not scored as an exchange, since it could be due simply to arm twisting at the kinetochore (Fig. 1 *a*). The total number of exchanges (Table II), when referred to the number of labeled chromosomes, is higher in third division metaphases than in second, because the former include exchanges belonging to both the second and the



FIGURES 1 *a* and 1 *b* *a*. Second division metaphase: All chromosomes have one labeled chromatid. Several exchanges (*E*) and a probable twist (*T*) at the kinetochore (rather than a sister chromatid exchange) are indicated by arrows.

b. Third division metaphase. Several chromosomes have only segments of a chromatid labeled because of sister chromatid exchanges that occurred during the preceding mitotic cycle (2nd cycle exchanges). A 3rd cycle exchange is indicated by the arrow.

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TABLE I

Fractions of Labeled Metaphases at Different Times after Labeling, as a Function of H^3-Thymidine Dose

Metaphases are classified according to the division, after labeling, to which they belong.

H^3-thymidine dose (μc/ml)	Hours after labeling	Unlabeled metaphases	Labeled metaphases (division)					1st plus 2nd metaphases
			1st	2nd	3rd	4th	Total	
								Per cent
0.54	37	105/300	1	56	87	51	195	29
0.54	45	147/300	0	16	75	62	153	10
0.54	62	191/300	0	0	5	104	109	—
0.80	37	92/300	2	67	97	42	208	33
0.80	45	137/300	0	30	75	58	163	18
0.80	62	174/300	0	2	20	104	126	2
1.20	37	133/300	0	101	35	31	167	60
1.20	45	154/300	0	12	51	83	146	8
1.20	62	299/300	0	0	0	1	1	—
1.80	37	178/300	5	89	16	12	122	77
1.80	45	235/300	2	31	13	19	65	51

TABLE II

Frequency of Exchanges in 2nd and 3rd Division Metaphases, as a Function of H^3-thymidine Dose

Metaphases were harvested approximately 37 hours after labeling.

H^3-thymidine dose (μc/ml)	Division after labeling	No. metaphases scored	Total No. chromosomes	Labled chromosomes (A)	Total No. exchanges (B)	Average No. of exchanges per labeled chromosome (B/A)
0.05	2nd	8	127	127	32	0.25
0.54	2nd	10	222	222	51	0.23
0.80	2nd	10	256	256	92	0.36
1.20	2nd	10	245	245	72	0.29
1.80	2nd	10	211	211	51	0.24
0.05	3rd	10	219	127	52	0.41
0.54	3rd	10	220	142	79	0.56
0.80	3rd	10	219	142	72	0.51
1.20	3rd	10	211	152	88	0.58

third cycles. Statistically the slopes of the curves in Fig.s 2 *a* and *b* are not significantly different from zero, and the frequency of exchanges, therefore, do not exhibit any detectable relation to dose over the range tested.

A more accurate estimate of exchange frequency per chromosome unit should be possible by eliminating the influence of the variation in chromosome number of individual metaphases. Therefore, the preparations were rescored for exchanges in the larger metacentric chromosomes only (chromo-I). In addition, since the effective radiation to which a chromosome is exposed is dependent on the uptake of H^3-thymidine, silver grains over each of the chromosomes were counted to obtain a better estimate of the actual radiation doses delivered. In Fig. 3 the average grain count per chromosome I is plotted *versus* concentration of H^3-thymidine in the medium for two sets of slides from the same experiment but coated with different batches of emulsion (see also Table II). A linear relation is evident in both sets of data (these do not match, probably because of uncontrolled variables in the autoradiographic procedures). The exchanges in chromosome I on the two sets of slides are given in Table III and are plotted in

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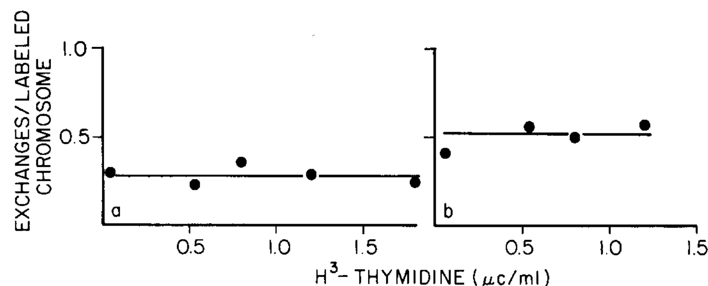

FIGURES 2 *a* and 2 *b* *a*. The data for 2nd metaphase sister chromatid exchanges given in Table II are plotted to show the relation between medium concentration of H^3-thymidine and the number of exchanges per labeled chromosome. The slope of the curve is not significantly different from zero.

b. Plot of 3rd metaphase exchanges against medium concentration of H^3-thymidine, taken from Table II. The slope of the curve is not significantly different from zero.

TABLE III

Frequency of Exchanges on Chromosome I in 2nd and 3rd Division Metaphases, as a Function of H^3-thymidine Dose

	2nd division metaphases				3rd division metaphases			
H^3-thymidine dose	No. chromosomes scored	Grains/ chromosomes/ hour	No. exchanges	Exchanges/ chromosome	No. chromosomes scored	No. of noncentromeric 2nd cycle exchanges	No. of centromeric 2nd cycle exchanges	No. 3rd cycle exchanges
(μc/ml)								
0.05	18	0.01	16	0.80	50	24	13	11
0.54	50	0.14	35	0.70	50	37	9	9
0.80	50	0.29	34	0.68	50	35	9	11
1.20	50	0.38	38	0.76	50	46	14	12
1.80	50	0.62	47	0.94	50	51	9	11
0.54	25	0.22	11	0.44				
0.80	25	0.38	20	0.80				
1.20	25	0.52	15	0.60				
1.80	25	0.92	19	0.76				
2.70	17	1.32	13	0.76				

Metaphases were harvested approximately 37 hours after labeling. Exchanges in 3rd division metaphases are classified according to the particular mitotic cycle in which they had taken place. Second cycle exchanges at the site of the centromere are listed separately. Data from two independent autoradiographic preparations of the same experiment are reported separately, but 3rd division metaphases were scored in only one case. Grains/chromosome/hour means the number of silver grains formed over a chromosome for each hour of exposure of the autoradiograph.

Figs. 4 *a* and *b*. The slopes of curves defined by these points are not significantly different from zero. The frequency of exchanges in chromosome I, therefore, does not bear any detectable relation to radiation dose over the dose range used.

The variation in grain counts for chromosome I from different cells treated with the same concentration (and same specific activity) was large. This is not surprising for an asynchronous population of cells exposed to H^3-thymidine for a time much shorter than the S period. Therefore the range of radiation doses to individual cells within each culture might have been even wider than the range of mean doses to which the different cultures were intentionally subjected. An estimate of this range is given in Table IV, where all the chromosomes scored are grouped for number of grains. Actually, the highest grain counts are lower than

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TABLE IV

Frequency of Exchanges on Chromosome I in 2nd Division Metaphases as a Function of the Number of Grains per Chromosome

Grains/chromosome/hour	No. chromosomes scored	No. exchanges	Exchanges/chromosomes
0.1 -0.2	12	5	0.42
0.2 -0.3	17	6	0.35
0.3 -0.4	19	15	0.79
0.4 -0.5	20	18	0.90
0.5 -0.6	12	6	0.50
0.6 -0.7	7	1	0.14
0.7 -0.8	10	4	0.40
0.8 -0.9	13	10	0.77
0.9 -1.0	13	8	0.61
1.0 -1.1	11	11	1.00
1.1 -1.2	12	6	0.50
1.3 -2.3	16	12	0.75
0.10-0.15	35	25	0.71
0.15-0.20	30	17	0.57
0.2 -0.3	33	23	0.70
0.3 -0.4	31	21	0.68
0.4 -0.5	28	14	0.50
0.5 -0.6	16	15	0.94
0.6 -0.7	12	16	1.33
0.7 -1.4	24	23	0.96

Metaphases were harvested approximately 37 hours after labeling. Column 1 gives the limits for classes of chromosomes having different grain numbers. Data from two independent autoradiographic preparations of the same experiment are reported separately.

TABLE V

Frequency of Exchanges on Chromosome I in 2nd Division Metaphases as a Function of X-radiation Dose

Metaphases were harvested approximately 35 hours after labeling.

H^3-thymidine dose	X-Radiation dose (r)	No. chromosomes scored	No. exchanges	Exchanges/chromosome
μc/ml				
0.5	0	30	15	0.50
0.5	25	50	31	0.62
0.5	50	50	38	0.76
0.5	100	50	40	0.80
0.5	200	50	41	0.82

the true value, since coincidence of grains must be high in the heavily labeled chromosomes. Nevertheless, the frequency of exchanges in each group of chromosomes. shows no significant increase with increase in labeling.

To test the effect of external irradiation on the frequency of exchanges, cells were cultured with 0.5 μc/ml of H^3-thymidine (1.5 c/mM) for 1 hour and then irradiated with various doses of x-rays. Colchicine was added 25 hours after labeling, and, 10 hours later, metaphases were harvested and fixed. The frequency of exchanges in chromosome I at different doses is given in Table V and Fig. 5. The positive slope of the curve defining the relation between number of exchanges and dose is significantly different from zero, indicating the possible induction of *some* sister chromatid exchanges by x-rays.

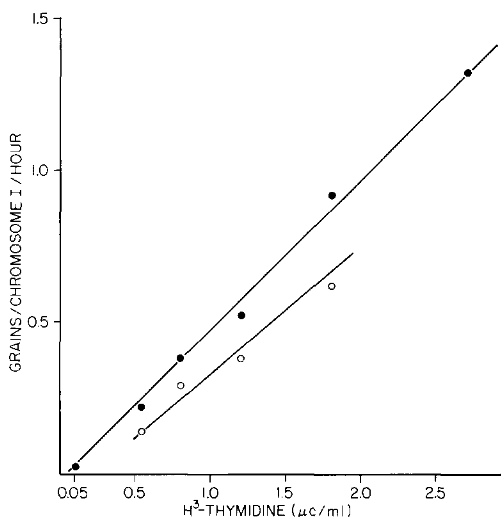

FIGURE 3 Average number of autoradiographic grains over chromosome I, as a function of H^3-thymidine concentration in the medium to which the cells were exposed. Fifty chromosomes were scored for each point.

DISCUSSION

It is generally assumed that damage following exposure of cells to medium containing tritiated nucleosides is due to β-radiation. A rigorous quantitative study of such a situation is difficult, because the actual radiation dose delivered to a cell for a given exposure to tritiated medium depends upon two parameters: (*a*) the amount of

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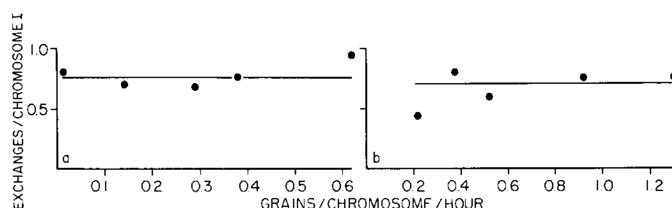
radioactivity in the cell from the time of uptake to the time of observation; (*b*) the rate of energy absorption as a function of the distance from the point sources of tritium. An estimate of (*a*) can be made only if the incorporation kinetics for the nucleoside are taken into account, and the absolute efficiency of the method adopted to measure uptake is known. As for (*b*), the estimation of the rate of energy loss for tritium β-radiation involves some uncertainty (13). Recently, Goodheart (4) calculated that the radiation dose per disintegration delivered to a nucleus containing a random distribution of point sources of tritium is 1.08 rad/disintegration.

At the higher exposures of H^3-thymidine used in our experiments, the amount of radiation delivered to the nuclei was sufficient to produce some significant lethality, and the results given in Table I indicate a selective advantage for the unlabeled cells. Both radiation dose estimates and the actual induction of cell killing indicate that chromosome aberrations are induced by the higher levels of H^3-thymidine; in fact, chromosome aberrations have been observed directly, in the same cell line, after comparable H^3-thymidine exposure (1). The frequency of sister chromatid exchanges, however, failed to show either a dependence on the amount of H^3-thymidine in the medium or a correlation with the actual degree of chromosome labeling.

Marin and Bender (6) measured the efficiency of our autoradiographic system on whole, flattened cells with H^3-thymidine and found a value of 14 disintegrations per autoradiographic grain formed.



FIGURES 4 *a* and 4 *b* Plots of the number of exchanges observed in chromosome I at the second metaphase against the average number of silver grains per chromosome per hour of autoradiographic exposure.

a. Taken from the upper set of data in Table III.

b. Taken from the lower set of data in Table III. Neither curve has a slope significantly different from zero.

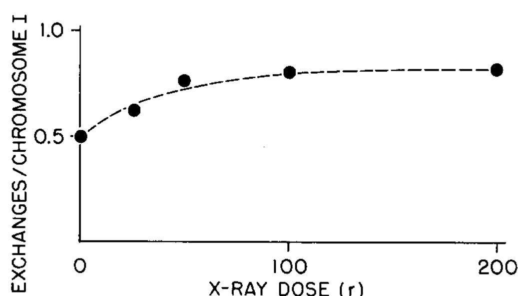


FIGURE 5 The number of exchanges per chromosome I is plotted against the number of r. The curve has a significantly positive slope initially but levels off after the 50 r point.

(The efficiency is probably very much higher in our studies, since absorption by cytoplasm was eliminated by isolating the chromosomes.) Using Goodheart's calculation of 1.08 rads/disintegration, this would mean 15 rads/grain formed. With the lowest incorporation, chromosome I produces 0.01 grain/hour. The pair of chromosomes I accounts for very close to 20 per cent of the total labeling, and the grains produced by the full complement of chromosomes is therefore about 0.05 per hour, or 0.75 rad/hour/complement. According to the studies of Marin and Bender (6), this radiation dose is an order of magnitude too low to produce any cell-killing. The same calculation for the highest incorporation of H^3-thymidine gives a value of about 100 rad/hour, a dose that should give some killing (3, 5). Since the frequency of sister chromatid exchanges does not change significantly between the two calculated doses of 0.75 and 100 rad/hour, we must conclude that if sister chromatid exchanges are radiation induced the exchange frequency must reach a maximum (saturation) well before the radiation dose can produce any cell killing and probably well before any detectable increase in visible aberrations occurs. This would indicate that sister chromatid exchanges are "spontaneous" events unrelated to radiation-induced chromosome breakage. Al-

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though this is our conclusion, there is still the formal possibility that the number of exchange sites is sharply limited and that at the lowest dose of tritium the frequency of exchanges has already reached the maximum. We are inclined to reject this explanation because of the relation between the calculated dose rate, cell killing, and induced aberrations discussed above.

Taylor's work on *Bellevalia* (14) is consistent with the above conclusion. In some of the experiments on *Bellevalia* the frequency of exchanges during the first full interphase after labeling (twin exchanges) was higher than during the second interphase (single exchanges); *i.e.*, after the radioactivity in each chromosome had been halved by division. This drop in exchange frequency might suggest that exchange induction is related to radiation. However, further experiments showed that the drop in exchange frequency might be due to the presence of colchicine during the second interphase, rather than to the change in radiation intensity.

In our experiments the exchange frequency remained the same over successive divisions. The exchanges listed under second division metaphases in Table III are those that occurred over the preceding two interphases (*cf.* reference 14). Therefore, the values listed under exchanges/chromosome are an accumulation for two interphases, and the true frequency/cycle is half of the average value for this column; *i.e.*, about 0.36 exchanges/chromosome/cycle. The third-cycle exchanges listed in Table III represent only half of the true value, since about half of the exchanges will occur in non-radioactive segments of chromosomes and be undetectable. Therefore, the exchange rate defined by the third-cycle exchanges is 21.5 exchanges per fifty chromosomes, or 0.43 exchanges/chromosome/cycle. The difference between 0.36 and 0.43 is not statistically significant.

Another point of interest here is the evidence of nonrandom distribution of exchanges over the length of chromosome I in CHEF-125 cells. From Table III it appears that one-fifth of all second-cycle exchanges scored in 3rd division metaphases took place at the centromere. (The frequency of second-cycle exchanges, not including centromere exchanges, scored in third division metaphases, corresponds to the value for second division metaphases, where exchanges at the centromere were deliberately not scored.) Centromeres did not appear to be "hot spots" of labeling; moreover, a higher concentration of radioactivity over a particular point of the chromatid does not necessarily mean a greater number of ionizations at that point. Assuming, therefore, a random distribution of radiation events over a labeled chromatid, any induced exchanges should be randomly distributed along the length of a chromatid

The data in Fig. 5 indicate that *some* sister chromatid exchanges may be caused by x-radiation. The increase in the number of exchanges over the range of 0 to 50 r is statistically significant, but there is no significant increase between 50 and 200 r. This suggests that saturation of exchanges may occur below 50 r. The high frequency of exchanges at zero r is considered to represent the spontaneous component. The chromosomes in these experiments have been labeled with tritium to make the exchanges visible autoradiographically, but, as indicated in the discussion above, we believe that the β-radiation from tritium has not significantly increased the exchange frequency.

In conclusion, x-rays appear to induce some sister chromatid exchanges but the increase is relatively small when compared with the increase of visible chromosome breaks over the same range of radiation intensities (2). The data taken *in toto* indicate a high spontaneous rate of sister chromatid exchange, and that exchanges are not induced by incorporated tritium or occur at too low a frequency to be detected against this high background.

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