[11] Wainwright, S. D., and A. Nevill, *J. Bacteriol.*, **70**, 547–551 (1955).
[12] Witkin, E. M., *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 123–140 (1956).
[13] Kimball, R. F., and T. Barka, *Expil. Cell Research*, 1959 (in press).
[14] Stapleton, G. E., D. Billen, and A. Hollaender, *J. Cellular Comp. Physiol.*, **41**, 345–357 (1953).
[15] Stapleton, G. E., A. J. Sbarra, and A. Hollaender, *J. Bacteriol.*, **70**, 7–14 (1955).
[16] Wolff, S., *Radiation Research*, Suppl. **1**, 453–462 (1959).
[17] Wolff, S., and H. E. Luippold, *Science*, **122**, 231–232 (1955).

*CHROMOSOME BREAKAGE PRODUCED BY TRITIUM-LABELED THYMIDINE IN TRADESCANTIA PALUDOSA**

By DONALD E. WIMBER

BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY

Communicated by Berwind P. Kaufmann, April 20, 1959

Introduction.—The original preparation of tritium-labeled thymidine by W. L. Hughes[1] and its application by Taylor, Woods and Hughes[2] in studies of chromosome duplication have led to wide-scale use of this labeled material in other areas of research. The fact that thymidine is a specific precursor of deoxyribonucleic acid (DNA) and that autoradiographs of very high resolution are obtainable using tritium have triggered most of these inquiries. Partial listings of the numerous investigations in which H^3-thymidine has been used are given by Cronkite *et al.*[3] and Hughes.[4]

Most of these studies have been concerned with the synthesis of DNA, the duplication, behavior and structure of chromosomes and the kinetics of cell populations, but relatively little research has been devoted to the possible biological effects of the internal soft beta radiations emitted by tritium. Although most workers have never doubted that some damage occurs, the extent of the damage has not been satisfactorily determined. Recently Plaut[5] has cautioned that use of H^3-thymidine in experiments may lead to erroneous conclusions because of the endogenous radiation. Painter, Drew, and Hughes[6] in a current paper have shown a marked inhibition of HeLa cell cultures grown on media containing 2.5 to 5.0 μc/ml of H^3-thymidine. Taylor[7] noted that fragments and interchromosomal exchanges were sometimes observed in squash preparations of *Bellevalia romana* roots that had been grown in nutrient solutions containing 2.5 to 5.0 μc/ml H^3-thymidine. In the present work chromosome fragmentation and mitotic inhibition are shown to occur in an organism treated with H^3-thymidine, and the degree of fragmentation will be related to amount of intranuclear irradiation measured by means of autoradiographs.

Materials and Methods.—Root tips of *Tradescantia paludosa* were used as experimental material. Plantlets were removed from the clonal parent (B2-2)[8] and the bases were placed in aerated Hoagland's solution under constant light (*c.* 400 ft. candles) and constant temperature (20° ± 1°C). After 72 hours most of the plantlets showed well developed roots.

Two procedures were used for labeling the nuclei. Some rooted plantlets were grown continuously in aerated nutrient solution containing 1 μc/ml H^3-thymidine

(0.36 C/mM supplied from Schwarz Laboratories, Mt. Vernon, New York) for 8 to 56 hours before fixing the root tips; others were grown in media containing 1 or 2 μc/ml for 4, 6, or 8 hours, washed thoroughly, transferred to isotope-free nutrient solution and fixed after 0 to 20 hours of further growth.

Roots were fixed in acetic-alcohol (1:3), hydrolyzed for 9 minutes in 1 *N* HCl at 60°C and stained by the Feulgen technique. Squashes were prepared in 45 per cent acetic acid and analyzed for fragments at anaphase. The squashes to be autoradiographed were frozen on dry ice, the cover slips removed and the slides placed immediately in 100 per cent ethanol. They were hydrated slowly and stripping film applied (AR 10 Kodak) according to the procedure outlined by Taylor.[9] The film was exposed for 24 hours and then developed. Activated silver grains over individual nuclei were counted.

Results and Discussion.—Mitotic figures in squashes from root tips grown in 1 μc/ml H^3-thymidine for 4 to 56 hours were scored for anaphase fragments. While aberrations were found in cells from roots in the labeling solution for 20 hours or more, it was soon apparent that there was a great variability of response of plantlets that had been grown in this solution for lengths of time over 8 hours; results from roots on a single plantlet seemed to show close agreement, but differences in frequencies of fragments between plantlets were sometimes rather great. These differences were thought to have arisen due to the variable number of roots on each plantlet and from the fact that a limited quantity of solution was available to each plantlet (10 ml); thus, the activity of those solutions with more roots would drop more rapidly than the average. However, if the data from several plantlets were grouped together, the results seemed to show a general trend. Through 16 hours few acentric fragments were noted, but from 20 to 24 hours there was a sharp increase in fragment frequency. There was then a drop to a relatively constant number of fragments which was maintained from 40 to 56 hours. These results are summarized in Table 1. The fact that the aberration frequency did not go

TABLE 1

THE EFFECT OF DURATION OF TREATMENT WITH 1 μc/ml H^3-THYMIDINE ON THE FREQUENCY OF ANAPHASE FRAGMENTS IN ROOT TIPS OF *Tradescantia paludosa*

Hours of Treatment	No. of Anaphases Analyzed	No. of Fragments	Fragments per 100 Cells
Control	1000	6	0.6
4	437	5	1.1
8	376	6	1.6
16	621	19	3.1
20	586	114	19.4
24	893	318	35.6
36	987	268	27.1
40	927	187	20.2
48	1625	307	18.9
56	980	200	20.4

higher after 24 hours could be attributed to a depletion of the H^3-thymidine in the nutrient solution. Scintillation counting for beta activity in the spent solutions in comparison to fresh isotope nutrient bore out this postulate. The presence of bridges, fragments, and micronuclei was similarly reported in root tip cells of *Allium cepa* grown in C^{14}-thymidine for extended lengths of time by McQuade, Friedkin, and Atchison.[10, 11]

In addition to an increase in fragment frequency, there was a drop in the mitotic index (cells in mitosis/total cells × 100) in roots that had been immersed in solutions of H^3-thymidine for over 8 hours. Normally the index was about 15 per cent in control roots; in some treated roots the index had dropped to 3 per cent.

In those preparations which had been scored previously for fragments and to which stripping film had been applied, it was possible to study fragment frequency as a function of grain density or extent of incorporation of H^3 into the chromosome. The number of grains over prophase, metaphase, anaphase, and telophase nuclei of roots grown from 24 to 56 hours in H^3-thymidine solutions was determined and the data were plotted against the number of fragments per 100 anaphase cells. The results are shown in Figure 1. Each point represents the combined data from several roots taken from one plantlet. The squashes selected for grain counting were purposely chosen for their high fragmentation in order that the percentage

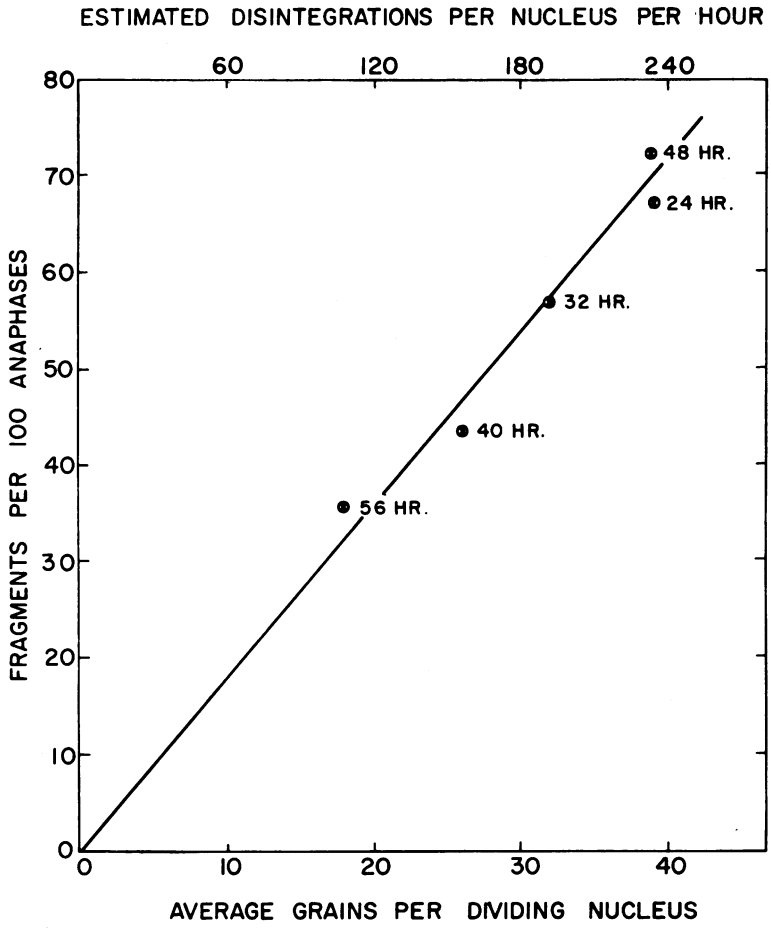

FIG. 1.—Increase in chromosome fragmentation in *Tradescantia paludosa* exposed to 1 μc/ml H^3-thymidine as a function of autoradiographic grain number over dividing nuclei. 200 or more cells were scored for each point. Disintegrations per nucleus calculated on basis of 1 grain per 143 disintegrations.

error in scoring so few cells would be at a minimum; thus, the average number of fragments per 100 cells found in Table 1 is always lower than those shown in Figure 1. The curve shows a roughly linear increase of grain count with degree of fragmentation. Within the limits covered by the observations the degree of fragmentation is proportional to the average amount of thymidine incorporated.

Delay of mitosis by intranuclear irradiation is indicated by the observation that nuclei that showed very high grain counts, i.e., 80 grains or more, were seldom found in division. Although nuclei were frequently observed with over 100 grains, they were almost without exception in interphase. For example, of 2145 interphases with more than 4 grains per nucleus, 11.5 per cent showed more than 80 grains over the nuclei; on the other hand, of 1512 labeled cells in division only 0.3 per cent showed over 80 grains. The difference is even greater than these figures indicate since the efficiency of the autoradiograph for nuclei in different stages must be taken into account. The average grain counts were about equal over all of the mitotic stages, i.e., prophase, metaphase, anaphase and telophase; furthermore, it was determined that cells in mitosis showed about one third more grains than cells in interphase. Thus, a grain count of 80 during mitosis would correspond to about 60 during interphase. Of the 2145 interphases examined, 15.9 per cent had more than 60 grains over the nuclei.

Because a number of workers have utilized roots that had been grown from 6 to 10 hours in H^3-thymidine (*c.* 2 μc/ml), it was deemed of interest to follow this same procedure and to look for fragments after further growth in isotope-free nutrient solutions for periods up to 24 hours. The results are reported in Table 2. The shorter periods of exposure of 4 to 8 hours in this experiment versus the longer

TABLE 2

CHROMOSOME FRAGMENTATION IN *Tradescantia paludosa* ROOT TIPS AS INDUCED BY EXPOSURE TIMES OF 4, 6 AND 8 HOURS IN 1 AND 2 μc/ml OF H^3-THYMIDINE

	1 μc/ml			2 μc/ml		
Collection Hour*	No. of Anaphases Analyzed	No. of Fragments	Frag./100 Cells	No. of Anaphases Analyzed	No. of Fragments	Frag./100 Cells
			4 hour exposure			
4	321	4	1.2	620	8	1.3
8	309	8	2.6	279	8	2.9
12	175	12	6.8	386	52	13.5
16	102	15	14.7	133	23	17.3
20	301	8	2.7	343	25	7.3
24	213	11	5.2	196	4	2.0
			6 hour exposure			
6	125	1	0.8	437	5	1.1
8	159	4	2.5	150	9	6.0
12	147	14	9.5	199	50	25.1
16	266	42	15.8	347	84	24.2
20	267	5	1.9	482	92	19.1
24	104	5	4.8	625	25	4.0
			8 hour exposure			
8	158	5	3.2	62	0	0.0
12	119	20	16.9	191	21	11.0
16	179	18	10.0	224	71	31.7
20	126	11	8.7	389	117	30.1
24	1060	25	2.4	589	81	13.7

* Zero hour is the time at which the plantlets were initially placed in the isotope solution. At 4, 6, and 8 hours the plantlets were changed to isotope-free solution and grown for various lengths of time up to the twenty-fourth hour.

periods of up to 56 hours in the other experiment minimized the interplantlet variation. Few fragments were found upon immediate examination of the root tips that had been immersed in the H^3-thymidine solution for 4 to 8 hours. However, a significant increase in the number of fragments was apparent when the root tips were allowed to continue division in isotope-free solution. For example, cells from roots that had been grown for 4 hours in 2 μc/ml H^3-thymidine, and then transferred to a nonisotopic solution for further growth showed a statistically significant increase in fragmentation at the twelfth and sixteenth hours from time of initial immersion into the isotope solution, i.e., 13.5 and 17.3 fragments per 100 cells respectively. Generally, there was a decrease in fragment frequency at the twentieth hour although data from roots that were treated for 6 and 8 hours in 2 μc/ml showed a slower decline than the data from those treated for a shorter time or with a lower isotope concentration, i.e., the fragment values were 19.1 and 30.1 per 100 cells for 6 and 8 hour exposures respectively; other values for the twentieth hour were less than half the above-mentioned figures.

From some other experiments that will be reported elsewhere it is known that the mitotic cycle in *Tradescantia paludosa* may be divided into the following average times and stages in relation to DNA synthesis:

(1) 4 hours, the period preceding DNA synthesis during interphase (G_1).
(2) 10 hours, DNA synthesis (S).
(3) 3 hours, non-DNA synthetic period before mitosis (G_2).
(4) 3 hours, mitosis (M).

These figures are similar to those reported for *Vicia faba* by Howard and Pelc.[12] Considering this time scale a simple estimate can be made of the length of time it will take for cells that had been in DNA synthesis during the total labeling period to reach anaphase, providing certain assumptions are made: namely, that the cells are all proceeding through the mitotic cycle at the same rate, that DNA synthesis is equally rapid during most of the synthetic period and that the precursor pool comes into equilibrium with the labeling solution very quickly. For those roots that had been grown in H^3-thymidine for 4 hours and then transferred to isotope-free nutrient, cells that had the maximum incorporation of this material would appear at anaphase between 9 and 15 hours; for the 6 hour sample, the most highly labeled anaphases would appear at 11 to 15 hours while in the 8 hour case they would be seen at 13 to 15 hours.

It is a reasonable expectation for those cells that absorbed the largest amount of H^3-thymidine to contain the greatest number of fragments at anaphase. The fragment data presented in Table 2 tend to confirm this hypothesis for there was an increase in fragmentation for the twelfth to the sixteenth hour and in some with the longer treatment the twentieth hour was involved. Even though at 24 hours most of the anaphases possessed few fragments, micronuclei were found to be rather common if the interphase nuclei were examined.

The findings presented above are of particular interest, since a number of researchers have utilized rather high concentrations (± 2 μc/ml) of H^3-thymidine for 6 to 10 or more hours. Taylor,[7] in particular, reported sister-chromatid exchanges at the second division after labeling *Bellevalia romana* root tips for 6 to 10 hours with 1.6 to 2.5 μc/ml of solution. He mentioned that these exchanges were possibly caused

by the endogenous radiation, but in a later publication[13] he seemed to think that their frequency was unaffected by the internal radiation. From the data herein presented it is evident that chromosome breakage is frequent; it is hardly necessary to conjecture that subchromatid damage would also be relatively common at comparable levels of activity and exposure times.

The abrupt rise in the number of chromosome fragments in the roots receiving continuous treatment (Table 1) occurred between 16 and 20 hours. In roots treated for a short time with the labeled precursor followed by growth in a nonlabeled medium (Table 2), the time of rise occurred generally much sooner, i.e., 12 to 16 hours. This delay in the rise of the aberration frequency may have been due to a cumulative radiation effect on the mitotic cycle. It is a well-known fact that one of the characteristic features of radiation damage is a delay in the onset of mitosis.

An estimation of the dose that each nucleus received from the endogenous decay of the tritium would be of interest but there are several obstacles that present themselves in calculating a correct dose. One of the foremost is the fact that the H^3 is confined almost entirely to the thymine of the DNA in the chromosomes. This could result in a nonrandom distribution of the tritium within the nucleus and also a nonrandom distribution of the beta rays.

One of the possible methods of estimating the dose is by a comparison of the fragment frequency with data from experiments with exogenous radiation. Mikaelsen[14,15] grew plantlets of the same clone of *Tradescantia paludosa* utilized here and exposed the roots for 48 hours to continuous gamma radiation from a Co^{60} source at an intensity of about 0.9 rad per hour. At the end of the treatment the average number of fragments per 100 cells at anaphase was reported as 22.1. Data given by Gray[16] indicated that β rays of the energy emitted by H^3 are about three times as effective per absorbed dose as γ rays in producing chromosome breaks. From Figure 1 roots exposed to H^3-thymidine for 24 hours show a fragment frequency of 67.5 per 100 cells. Although no direct comparison of these findings may be made to Mikaelsen's data, the dose rate delivered to the nuclei by the H^3 in this case is probably no more than five times that of the gamma intensity, i.e., less than 4.5 rads per hour.

Theoretically a rather accurate assay of the dose delivered to each nucleus could be made on the basis of grain counts over the nuclei. Hughes and co-workers[17] have made some estimates of the efficiency of grain counts in assaying the total number of disintegrations per nucleus. They judge that in a tissue section about one grain is formed per 50 disintegrations over nuclei that have been cut in two. The real value is probably somewhere between 10 and 100 disintegrations per activated grain. Although the actual steps will be omitted here, it may be calculated that an average grain number of 36.6 over dividing nuclei in a squash preparation produced by a film exposure of 24 hours would deliver to the nuclei between 5.2 and 52 rads per hour. Expressing it another way, roughly one anaphase fragment would result from 64.6 to 646 disintegrations per hour per 100 nuclei at the time of fixation; this estimate assumes a direct functional relationship between fragment frequency and autoradiographic grain number. In Figure 1, where grain count is plotted against fragment frequency per 100 cells, a grain count of 36.6 can be correlated with an induced fragment frequency of 67.5 per 100 cells. The estimates of dosage based on two different criteria can now be compared, i.e., 5.2—52 rads/hour versus <4.5 rads/hour.

Certainly the lowermost calculation based on grain count is the most compatible with the estimate taken from Mikaelsen's data. But, until more accurate measurements of tritiated substances in producing autoradiographs are available, we shall probably be unable to make more accurate estimations of the actual dose received.

Summary.—The fragmentation of chromosomes in the root tips of *Tradescantia paludosa* as produced by tritium-labeled thymidine was measured. As many as 31 fragments per 100 cells occurred in roots grown for 4 to 8 hours in 1 or 2 μc/ml H^3-thymidine and then transferred to isotope-free solutions for further growth before collection. The highest fragment frequency could be correlated with the period during which the maximum amount of H^3-thymidine was incorporated into the chromosomes. Up to 72 fragments per 100 cells at anaphase were demonstrated after continuous exposure of root tips to 1 μc/ml H^3-thymidine for times up to 56 hours.

The mitotic index dropped sharply in roots that had been exposed to 1 μc/ml of H^3-thymidine for periods over 8 hours. Cells that absorb large quantities of H^3-thymidine as indicated by autoradiographs are seemingly disturbed in their development and are delayed or prevented from entering mitosis. Autoradiographs of roots that had been exposed to 1 μc/ml H^3-thymidine for 24 to 56 hours showed that the average grain count over dividing nuclei increased as the fragmentation appearing at anaphase increased.

Approximations of the dose delivered to the nuclei by the endogenous radiation from the tritium were made by two different methods; however, the estimates showed poor agreement. Confident calculations of the dose probably await the determinations of the efficiency of the β rays in producing an autoradiograph.

These findings would indicate that investigators utilizing tritiated substances should interpret their results with caution for the endogenous radiation delivered to a cell may be great enough to cause considerable chromosome breakage and presumably other forms of genetic damage as well as significant changes in cellular physiology.

The author gratefully acknowledges the advice of Dr. A. H. Sparrow during the course of the work and discussions with Drs. P. S. Woods, D. Steffensen, H. Quastler, and Miss V. Pond during the preparation of the manuscript.

* This investigation was carried out while the author was a Public Health Service Research Fellow of the National Cancer Institute (CF-8465) at the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[1] Hughes, W. L., unpublished work at Brookhaven National Laboratory, Medical Department, Upton, New York.

[2] Taylor, J. H., P. S. Woods and W. L. Hughes, these PROCEEDINGS, **43**, 122 (1957).

[3] Cronkite, E. P., V. P. Bond, T. M. Fliedner, and J. R. Rubini, *Lab. Invest.*, **9**, 263 (1959).

[4] Hughes, W. L., in *A Symposium on the Chemical Basis of Development*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1958), p. 136.

[5] Plaut, W., *Lab. Invest.*, **9**, 286 (1959).

[6] Painter, R. B., R. M. Drew, and W. L. Hughes, *Sci.*, **127**, 1244 (1958).

[7] Taylor, J. H., *Genetics*, **43**, 515 (1958).

[8] Clone B2-2, a selection from selfing Sax's clone 5.

[9] Taylor, J. H., in *Physical Techniques in Biological Research*, ed. G. Oster and A. W. Pollister (Academic Press, 1956), p. 545.

[10] McQuade, H. A., M. Friedkin, and A. A. Atchison, *Nature*, **175**, 1038 (1955).

[11] McQuade, H. A., M. Friedkin, and A. A. Atchison, *Exp. Cell Res.*, **11**, 256 (1956).
[12] Howard, A., and S. R. Pelc, supplement to *Heredity*, **6**, 261 (1953).
[13] Taylor, J. H., in discussion of W. Plaut's paper in *Lab. Invest.*, **9**, 293 (1959).
[14] Mikaelsen, K., *Sci.*, **116**, 172 (1952).
[15] Mikaelsen, K., these PROCEEDINGS, **40**, 171 (1954).
[16] Gray, L. H., *Rad. Res.*, **1**, 189 (1954).
[17] Hughes, W. L., V. P. Bond, G. Brecher, E. P. Cronkite, R. B. Painter, H. Quastler, and F. G. Sherman, these PROCEEDINGS, **44**, 476 (1958).

---

*ENZYME COMPLEMENTATION IN VITRO BETWEEN ADENYLOSUCCINASELESS MUTANTS OF NEUROSPORA CRASSA**

BY DOW O. WOODWARD

DEPARTMENT OF BOTANY, JOSIAH WILLARD GIBBS RESEARCH LABORATORIES, YALE UNIVERSITY

Communicated by Edmund W. Sinnott, April 24, 1959

Previous studies[1] have shown that certain mutant strains of *Neurospora crassa* lacking detectable adenylosuccinase activity, and hence unable to grow in the absence of exogenous adenine, complement one another in certain combinations when grown as heterocaryons. Such complementation results in adenine-independent growth, and certain of the resulting heterocaryons possess a maximum of 25 per cent of wild-type adenylosuccinase activity. The pattern of complementation has been depicted as a complementation map of the *ad-4* locus and suggests a structural correlation between gene and gene product.[2-4] These results led to the hypothesis that complementation involves a cytoplasmic exchange mechanism operating at either the RNA or polypeptide level. The work of Singer and Itano,[5] demonstrating the formation of hybrid protein *in vitro*, lends further support to the suggestion that exchange at the polypeptide level could provide a mechanism for complementation. In addition, such a mechanism could also explain the restoration of enzyme activity in heterocaryons to a maximum level not exceeding 25 per cent of wild type. Other recent studies[6-9] suggest related types of exchange mechanisms that could also yield hybrid protein or protein complexes.

The above hypothesis has received additional support with the demonstration of *in vitro* complementation. By mixing mycelial homogenates from certain pairs of complementing adenylosuccinaseless *Neurospora* mutants grown separately, adenylosuccinase activity as high as 20 per cent of that obtained from *in vivo* complementation has been recovered. The same procedure performed with complementing mutants, singly, or with mixtures of non-complementing mutants, has failed to restore detectable enzyme activity. Earlier attempts[1] to detect enzyme activity in mixtures of extracts from complementing *ad-4* mutants gave negative results.

Materials and Methods.—Data on the origin and other characteristics of the *ad-4* mutants employed in these studies have been presented.[4]

The following general procedure has been found to be the most effective in demonstrating *in vitro* complementation in mixing experiments. Mutant cultures are grown separately for 3–4 days at 25°C on Fries minimal medium containing 100