The Effect of HCl Hydrolysis on the Retention of Thymidine in DNA*

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ABSTRACT

Allium roots grown in C^{14}-thymidine and H^3-thymidine media were treated with N hydrochloric acid at 60°C. as in standard Feulgen hydrolysis. The retention of the radioactive thymidine in DNA as a function of hydrolysis time was studied autoradiographically. No significant loss of label was detected until hydrolysis was extended beyond the optimal time for Feulgen staining. The data are consistent with the assumption that there is no significant loss of DNA during normal Feulgen hydrolysis in the material used.

Recently it has been suggested (Woods, 1957) that acid hydrolysis optimum for Feulgen staining of cellular deoxyribose nucleic acid (DNA) results in the loss of nearly two-thirds of the DNA. For this study, anthers of *Lilium longiflorum* ("Croft") were frozen and dehydrated according to the method of Woods and Pollister (1955), fixed in hot 75 per cent alcohol, and hydrolyzed with 10 per cent perchloric acid at 20°C. for periods of time ranging from 15 minutes to 144 hours. The liberation of thymine into the hydrolysates at these time periods was taken as the criterion of DNA loss.

The implications of this suggestion to general quantitative cytochemistry of DNA are sufficiently important to warrant reinvestigation of the problem under the standard conditions of the Feulgen procedure. Such a study is reported in this paper. *Allium* root tips were fixed in alcohol-acetic acid mixture, and hydrolysis was carried out with hydrochloric acid. Changes in DNA content of the root tips as a function of hydrolysis time were followed by the use of radioactive isotopes.

Material and Methods

Allium sp. set bulbs, placed on vials of distilled water, were allowed to germinate roots during 4 days. Two bulbs, each with twenty to thirty roots, were then placed on separate vials with their roots in contact with White's inorganic medium. One of the vials contained approximately 0.24 microcuries C^{14}-thymidine per milliliter of White's medium, and the other contained approximately 0.32 microcuries of 55 per cent radiopure H^3-thymidine (Schwarz Laboratories, Inc., Mount Vernon, New York) per milliliter of solution. Roots were grown in these media for 72 hours, rinsed with distilled water, fixed for 2 hours in 3 parts ethanol: 1 part glacial acetic acid, and hydrolyzed for lengths of time varying from 0 minutes to 20 minutes in 1 N HCl at 60°C. Squashes in 45 per cent acetic acid were made on microscope slides treated with egg albumen. The tissue on the slides was quick-frozen in liquid air, the coverslips removed, and the slides placed in absolute alcohol. The slides were passed through increasing dilutions of alcohol and finally rinsed three times in distilled water. The tissue was covered with stripping film (Kodak plate "autoradiographic" stripping film Kodak, Ltd., London) after the method of Pelc (see Doniach and Pelc, 1950). Films were exposed for 11 days in the dark under refrigeration (5–7°C), developed with Kodak D-19b, and fixed with Kodak F 5.

All autoradiographic slides were examined with a Leitz ultropak objective lens fitted with a phase ring, enabling location of cells by phase contrast microscopy and counting of emulsion silver grains by reflected light. The number of silver grains reduced over a prophase or metaphase figure was counted at × 460 magnification. When the number of grains above one nucleus was counted two or three times, the reproducibility of counts was found to be good (6 per cent error or less). The numbers given in Tables II and III represent averages where more than one count was made.

There was considerable variability in the number of silver grains above nuclei of identical stage and treatment. The exposure of the roots to the radioactive media for as long a period as 72 hours had been an at-

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TABLE I

The Percentage of Nuclei Which Have a Number of Silver Grains Equal to or Greater than Five Times the Background*

H^3-thymidine		C^{14}-thymidine	
Time of hydrolysis	Nuclei	Time of hydrolysis	Nuclei
min.	*per cent*	*min.*	*per cent*
1	29.2		
2	17.9	1	17.1
4	13.6	2	22.6
6	29.4	4	18.2
8	52.9	8	21.4
10	38.9	8	14.3
15	15.9	(a second slide)	
		10	25.7
		15	17.4

* The term "nuclei" as used here and in the other tables refers to chromosome sets regardless of the presence or absence of a nuclear membrane.

tempt to eliminate this anticipated variability. Since in a squash preparation of a root tip it is impossible to ascertain whether or not a given cell is of meristematic tissue or of root-cap, epidermal, or other nonmeristematic tissue in which mitosis and DNA synthesis would be expected to be infrequent and irregular, it was decided that the number of silver grains over only prophase and metaphase figures would be examined. Since variability was also great over these two stages it was necessary either to count an exceedingly large number of cells or to carry out a sampling of them. The former possibility was eliminated because of the limited number of mitotic cells in the roots and the amount of time which would have been necessary to make such counts. The second course was thus followed, and only those instances in which the number of silver grains over a mitotic figure exceeded the background number by five times were counted. If hydrolysis resulted in a loss of DNA, it could be argued that such a loss would not be detected by this sampling method since it selects cells above a certain labelling intensity. This argument would be valid if the percentage of cells labelled above the selected intensity decreased with hydrolysis time. An examination of the slides of each hydrolysis time was therefore made to ascertain whether or not there was a drop in this percentage. As shown in Table I, the results indicate that the percentage of such highly labelled cells varies randomly without correlation to the length of hydrolysis. Thus the sampling procedure employed is not likely to have influenced the results significantly.

RESULTS

The effect of acid hydrolysis upon retention of C^{14}-thymidine and H^3-thymidine by *Allium* root cells is shown in Tables II and III. In Table II (C^{14}-thymidine data), each column represents the number of silver grains reduced in the emulsion over mitotic figures found in two roots except for the Prophase 15 Minute Hydrolysis column, and the Metaphase 0, 2, 8, and 15 Minute Hydrolysis columns, for each of which only one root was available for analysis. In Table III (H^3-thymidine data), each column also represents counts made on two roots except for the Prophase 2 Minute Hydrolysis column, the Metaphase 0 and 2 Minute Hydrolysis columns, for which three roots were available, and the Metaphase 6 Minute Hydrolysis column, for which only one root was used.

The relatively small number of entries in the columns of 0 Hydrolysis time in both treatments results from the difficulty in locating recognizable mitotic figures in the tissue which had been badly disrupted as a result of squashing without prior hydrolysis.

The unidentified contaminant amounting to 45 per cent of the radioactivity in the H^3-thymidine preparation is not incorporated into a non-DNA component of the cell: all of the radioactivity remaining in the tissue after fixation could be removed by digestion with deoxyribonuclease.

DISCUSSION

The results of this study are consistent with the findings of Thomas (1950) that there is no significant liberation of thymine from DNA until after 12 minutes of hydrolysis at 60°C. with 1 N HCl. The data do not, therefore, support the contention that there is substantial loss of thymine during optimum hydrolysis for Feulgen staining which, for onion roots, is 8 to 10 minutes with 1 N HCl at 60°C. The summed data of the C^{14}-thymidine experiment indicate the absence of significant loss of labelled thymidine until after 10 minutes of hydrolysis, and a more or less complete loss by 20 minutes. These results are essentially confirmed by the summed H^3-thymidine data, although in this case there is the possibility of some loss in the 10 minute hydrolysis sample. The greater heterogeneity of the tritium data suggests that this drop may be attributed in part at least to one or another of the causes of variability discussed below.

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TABLE II

C^{14}-Thymidine

	No. of silver grains reduced in emulsion over nuclei							
	Time of hydrolysis							
	0 min.	1 min.	2 min.	4 min.	8 min.	10 min.	15 min.	20 min.
Prophases								
	101.7	95.0	147.7	188.0	166.3	92.0	106.5	
	80.0	99.5	30.0	72.0	170.5	155.0	76.5	No
	109.0	78.5	110.0	76.5	122.0	106.0	40.5	label
	156.0	90.5	90.0	106.3	77.7	78.0	77.0	
		195.0	51.0	167.0	127.5	37.5	37.5	
		224.5	92.5	59.5	59.3	126.5	138.5	
		203.0	111.0		167.0	191.5		
		101.5			94.0	290.0		
		72.5				193.5		
						146.0		
						92.5		
Mean	111.7	128.9	90.3	109.9	134.5	143.2	79.4	0.0
Standard error	16.0	20.1	14.9	23.9	15.3	18.9	15.8	—
Metaphases								
	174.7	128.5	167.5	183.0	139.0	105.7	150.0	
		57.0	165.7	91.0	128.7	113.0	38.5	
		82.0	52.7	79.0	83.0	110.0	32.5	
		79.5	67.0	144.0	121.5	52.0	140.5	No
		233.5	145.0	177.5	256.0	133.3	44.5	label
		98.5	76.0	83.0	187.5	161.5	64.0	
		237.0	101.0	144.0	118.0			
		190.0		101.3				
Mean	174.7	138.3	110.7	125.4	146.7	112.6	78.3	0.0
Standard error	—	15.5	18.3	14.9	22.0	14.8	21.6	—
Prophase and Metaphase Data Summed								
Mean	124.1	133.3	100.5	119.4	134.5	132.4	78.9	0.0
Standard error	17.7	15.5	11.7	12.3	12.9	13.5	13.1	—

Average background count: 11.1 grains per nucleus.
Range of background counts: 0 to 28 grains per nucleus.

The observations do not support the suggestion (Woods, 1957) that there may be a significant amount of cytoplasmic DNA. Within the limits of autoradiographic resolution, labelling was confined to nuclei or mitotic figures. One must thus assume that either there is no DNA present in the cytoplasm or that if there is such DNA, it is either readily lost from the cell during fixation, or its synthesis is such as not to lead to labelling with thymidine in the course of an experiment such as this.

The results indicate considerable variability in the number of silver grains reduced in the emulsion over radioactive nuclei of a single mitotic stage.

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TABLE III

H^3-Thymidine

	No. of silver grains reduced in emulsion over nuclei								
	Time of hydrolysis								
	0 min.	1 min.	2 min.	4 min.	6 min.	8 min.	10 min.	15 min.	20 min.
Prophases									
	96.0	140.0	166.5	74.3	39.3	123.0	40.3	80.7	
	85.0	70.0	73.0	88.3	43.7	193.5	32.7	91.5	No label
	51.0	123.0	73.0	76.3	45.0	208.3	38.7	38.5	
	65.0	91.0	72.5	120.0	33.3	138.7	84.0	27.0	
	69.0	231.0	74.0	49.0	108.5	57.0	125.3	29.7	
	119.3	83.0	348.0	194.5	127.5	77.3	221.0		
		77.0	343.0	98.5	189.0	71.0			
		48.5	100.0	197.0	148.0	96.5			
		44.0	125.0		127.5	115.5			
		79.0			162.0	149.5			
		133.0			54.5	134.5			
		79.0							
		112.0							
Mean........	80.8	100.9	152.8	112.3	98.0	124.1	90.3	53.5	0.0
Standard error.	10.0	13.6	41.4	19.6	17.0	14.5	16.4	13.5	—
Metaphases									
	318.0	67.3	119.5	98.0	189.0	161.0	52.0	63.0	
	331.0	278.0	52.0	290.0	111.0	162.5	110.7	67.7	No label
	126.5	145.5	163.5	182.0	141.0	55.3	107.3	15.0	
	58.3	109.5	174.0	64.7	107.5	57.0	36.0		
	149.5	53.3	140.5			121.5	170.5		
		92.5	126.5			118.5	153.0		
			255.0			64.0			
			241.0			169.0			
Mean........	196.7	124.4	159.0	158.7	137.1	113.7	103.3	48.6	0.0
Standard error.	45.4	33.5	23.4	50.3	18.9	17.4	21.7	16.9	—
Prophase and Metaphase Data Summed									
Mean........	133.5	108.3	155.7	127.8	108.5	119.7	96.8	51.6	0.0
Standard error.	29.7	13.8	22.2	21.5	13.9	10.9	17.8	9.8	—

Average background count: 8.4 grains per nucleus.
Range of background counts: 0 to 42 grains per nucleus.

This variability is evident not only among several roots of identical treatment, but also among the cells of one root. Variability was greater with H^3-thymidine than with C^{14}-thymidine. The most extreme case can be seen in the roots hydrolyzed for 2 minutes (see Table III). The mean of the standard errors of the means in the H^3 experiment is 23.3, while in the C^{14} experiment it is 17.8. Factors which may account for this variability include the problem of the geometric relation of the radioactive material to the emulsion. The importance of this problem is especially great with

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tritium, for the range of the H^3 beta particle is small. The consistent observation that more silver grains occur over flattened nuclei of large exposed areas than over rounded nuclei of small exposed areas supports this factor as a cause of variation.

Another probable cause of greater variability among the tritium data is the fact that H^3-thymidine was less uniformly available over the period of the experiment to the cells of the roots than was the C^{14}; the specific activities of the H^3 and C^{14} media were about 150 microcuries per micromole and 4 microcuries per micromole respectively; hence the tritium medium had less thymidine than the C^{14} medium as the radioactivity per milliliter was approximately equal. Furthermore, a decrease in the radio-activity per milliliter of the tritiated thymidine medium over the period of such an experiment is readily demonstrable. As a consequence, cells synthesizing at the end of the period of exposure to the tritium medium had less radioactive thymidine available to them than did those cells synthesizing at the start of the period.

Other factors which may account for variability in all data include the possibilities of occasional polyploidy, of differences in cycle of DNA synthesis in different nuclei, and of differing lengths of time in the labelled thymidine medium (a few roots may have been initiated during the 3 day growing period). All of these factors would be expected to be randomly distributed, however, and hence should not effect the over-all results.

The inconsistency between the results reported here and those of Woods may be attributed to differences in the experiments themselves. These differences include the choice of tissue, the methods of dehydration and fixation, the acid and temperature used in hydrolysis, and the means of detecting changes in DNA content of the tissues.

A criticism of the use of *Lilium* anthers, with the method employed by Woods for following changes in tissue DNA, stems from the peculiar nature of some of the tissues of the anther. It has been observed that during the course of the development of pollen grains, the tapetal layer, which lines the inner surface of the locule, undergoes degeneration (see Mechelke, 1952). This degeneration is particularly noticeable during the late meiotic and postmeiotic periods of the developing microsporocytes, which are the stages found in the anthers of *Lilium* buds of the length chosen by Woods for his study (see Erickson, 1948; Taylor and McMaster, 1954). Furthermore, there have been observations of Feulgen-positive extrusions from tapetal cells (Cooper, 1952) which have been shown by Takats (1957) to be a rapid response to mechanical disruption of the anther. Takats has also shown that the microsporocytes themselves extrude Feulgen-positive globules in the natural course of their development, and although his findings place this event at a stage earlier than normally found in *Lilium* buds 24 to 25 mm. long, it is not inconceivable that there may be remaining in the locule products from these extrusions. There exists, then, the possibility of DNA degradation products within the anther which are not intrinsically bound to the cells and which could be removed by less drastic treatment than hydrolysis. It should be pointed out, however, that the anthers in Woods' experiment were treated prior to hydrolysis with cold 2 per cent perchloric acid (4°C.) for 40 minutes to remove from the tissue "purine and pyrimidine containing materials other than nucleic acids." Nevertheless, it is conceivable that this treatment might not remove degraded or partially degraded DNA products which are yet weakly bound to cellular constituents or trapped within the anther locules, but which treatment with 10 per cent perchloric acid at 20°C. for up to 19 hours may free.

The method of dehydration and fixation in Woods' experiment consisted of freezing the anthers in partly frozen isopentane, dehydrating them in absolute alcohol at $-41°$ to $-45°$C. over a period of 3 days, and postfixation in 75 per cent alcohol at 63°C. for 2 hours. Although Feulgen staining after these procedures is shown by Woods to be equivalent to Feulgen staining after the more conventional acetic-alcohol fixatives, it would be impossible to conclude from this evidence that the glycosidic bonding of thymine is unaffected by the treatment (see also Taylor, 1958).

In Woods' experiment, hydrolysis was effected with perchloric acid. The Feulgen staining pattern after perchloric acid hydrolysis has been shown by DiStefano (1952) to be equivalent to the pattern after HCl hydrolysis. Again, this equivalence does not preclude the possibility that the two acids affect the thymine bonding differently; it has already been suggested (Taylor, 1958) that the use of perchloric acid may account for early liberation of thymine in Woods' experiment.

Although the different means employed in these two studies for following DNA changes in the tissue do not in themselves account for differences in results, the autoradiographic method has an advantage in that it permits a direct analysis of

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DNA remaining in the tissue. Conclusions concerning cellular DNA derived from chromatographic examination of the tissue hydrolysates are necessarily indirect.

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