^{3}H-Thymidine Incorporation into Nuclear DNA of Leaf Cells[1]

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Summary. The incorporation of ^{3}H-thymidine into nuclear DNA of leaf cells of *Xanthium pennsylvanicum* was studied as a function of concentration and specific activity of the radioisotope. From the assessment of the average number of grains per nucleus and the percent of labeled nuclei, it was concluded that the incorporation was a linear function of concentration of the exogenous radioisotopic solution and a logarithmic function of the incubation time. Ten microcuries per milliliter on the average yielded 20 % of labeled nuclei with 18 grains per nucleus. Seven-fold increase in concentration only doubled the amount of ^{3}H-thymidine incorporated. The lamina regions near the vein incorporated a significantly greater amount of the radioisotope than the lamina region at some distance from the vein. The specific activities of 2, 3.35, 6.7 and 15.3 c/mmole had no effect upon the amount of ^{3}H-thymidine incorporated, if the amount of microcuries of the incubation solution was the same in each activity. Considering the total number of molecules, the estimated rates of incorporation indicated that at the activity of 2 c/mmole, the system operated with about 7 times higher rates as compared with the activity of 15.3 c/mmole.

The application of ^{3}H-thymidine in developmental studies and investigations dealing with the activity of various plant meristems has increased extensively [6, 8, 3, 11, 12]. However, little quantitative information is available concerning the kinetics of incorporation of this radioisotopic precursor into nuclear DNA of various plant tissues. In the labeling procedures the concentration and the specific activity remain the most important variables in addition to the isotopic and postisotopic growth periods.

The purpose of this investigation is to explore the effect of ^{3}H-thymidine concentration and its specific activity upon incorporation of this radioisotope into nuclear DNA. In addition, an attempt has been made to estimate the rates of incorporation and the number of thymidine and ^{3}H-thymidine molecules incorporated into DNA per nuclear volume as a function of the specific activity and molar concentration.

Materials and Methods

Burs of *Xanthium pennsylvanicum* Wallr. (Synonym *X. saccharatum*) were germinated in flats of soil. After the seedlings had developed the first 2 foliage leaves, they were planted in pots. The plants were grown in a walk-in-type growth chamber where the light intensity fluctuated between 600 and 700 ft-c depending upon the height of the plant. The temperature was adjusted to 25° ± 0.5. The relative humidity was maintained at about 50 %. To maintain vegetative growth the illumination was cycled to give incandescent light period of 18 hours and a dark period of 6 hours. Leaves on the primary shoot were numbered in order to their appearance and the studies were made with the middle portion of leaf nine. The morphological ages of leaves were determined by use of the leaf plastochron index (L.P.I.). Leaves used in this investigation were selected at about L.P.I. 0.5. At this stage the leaf lamina is characterized by 6 compact meristematic layers of cells and active cell division. The duration of 1 L.P.I. was on the average 3.4 days for the plants grown under specified environmental conditions. For estimation of plastochron ages (P.I.) of plants and leaves, the reader is referred to Erickson and Michelini, and Erickson (4, 5).

The nuclear volume was estimated from fixed and sectioned tissue from camera lucida drawings at various plastochronic ages of leaves. Nuclei which approximated a sphere were selected for this purpose.

Methyl labeled ^{3}H-thymidine was incorporated by a method of foliar absorption of the isotope. In this method, the intact *Xanthium* plants were set in an inverted position. The whole shoot with young leaves was submerged in 0.1 % Tween for about 15 minutes to facilitate uptake of the isotope through the epidermal waxes (9). The wetting agent was replaced with the isotope for 4 hours. Air was bubbled into the medium to increase oxygen concentration. The time of growth in the isotope is referred to as the isotopic growth. After the isotopic growth, the leaf was rinsed with distilled water, and the plants were grown for 4 hours in the absence of the ^{3}H-thymidine to allow time for translocation and incorporation. This period is re-

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ferred to as the postisotopic growth. The tissue was cut into 4 μ sections and stained with Feulgen. Kodak Nuclear Track Emulsion NTB-2 was used in autoradiography. Slides were exposed for 2 weeks at 4° and processed according to established autoradiographic techniques (8). A Bausch and Lomb Whipple micrometer was used for grain counts and estimation of the percentage of labeled nuclei in the lamina region between 2 veins. A series of DN-ase experiments were performed to check on the specificity of ^{3}H-thymidine incorporation into nuclear DNA.

The term average number of grains per labeled nucleus as used throughout the text should be interpreted as the average number of grains per labeled nuclear section. Similarly, the percent of labeled nuclei in actuality means the percent of labeled nuclear sections as seen on a slide. In contrast, referring to the nuclear volume, the estimated number of ^{3}H-thymidine molecules and also the amount of microcuries incorporated will be expressed per 74 μ^3 of the average labeled nucleus.

The analysis of data at various isotopic growth periods and various specific activities are illustrated in table I. The average number of grains per nucleus was estimated from leaves treated with the isotope of various specific activities and concentrations expressed as c/mmole and μc/ml, respectively. To consider a nucleus as labeled, a minimal number of grains per nucleus was established which was significantly higher than the background of the same area. The range of background radiation was between 0.2 and 0.4 grains per nucleus. Nuclei with 6 grains or more were considered as significantly labeled. The straight lines in figure 3 were fitted by least squares method, but the curves in figure 4 were fitted by eye. To indicate the variability of data the 95 % confidence intervals were calculated. The rates of incorporation (dX/dt) were calculated using 3-point formula for numerical differentiation (13).

Table I. *Incorporation of ^{3}H-Thymidine into Nuclear DNA*

Isotopic growth	Sp act	Nuclei scored	Grains/ nucleus	Confidence Intervals
Hrs	c/mmole	Total no	Avg no	±
	2.00	846	7.85	1.26
1	3.35	852	8.76	1.00
	6.70	1080	9.67	1 68
	15.30	990	9.23	1.72
	2.00	1736	18.10	1.60
4	3.35	733	15.91	1.59
	6.70	890	17.05	1.69
	15.30	778	20.77	2.14
	2.00	663	20.95	1.86
8	3.35	488	16.60	1.70
	6.70	615	18.65	1.72
	15.30	712	24 02	3.45

Results and Conclusions

In quantitative autoradiography perhaps the most useful approach in collecting data is the assessment of the average number of grains per nucleus and the estimation of the average percent of labeled nuclei. Both of these variables indicate a relative amount of the radioactive precursor incorporated into DNA molecules. The percentage of labeled nuclei will represent a relative number of cells in a cell population engaged in DNA synthesis. The number of grains per nucleus however, will be indicative of the amount of the radioactive precursor incorporated into nuclear DNA of a single cell.

The average percent of labeled nuclei in figure 2 increases linearly up to about 16 hours of isotopic growth. This means that the rate of change in number of cells engaged in DNA synthesis during this phase is constant. Further curvature of the lines indicates a gradual decrease in the rate of incorporation, and 48 hours of isotopic growth give about 90 % of labeled nuclei. Judging by the confidence intervals, the lamina region near the vein, represented on the graph by the dashed line, gave a significantly higher percentage of labeled nuclei than the lamina region at some distance from the vein. The average number of grains per nucleus of the near-vein region was also significantly higher than that of the lamina proper. One possible explanation is that not all of the radioactive precursor initially absorbed by the lamina cells was immediately incorporated into nuclear DNA. Some of this precursor may have been translocated into the vascular system and additionally made available to the dividing cells located near the veins. The near-vein region may be a metabolically distinct part of the

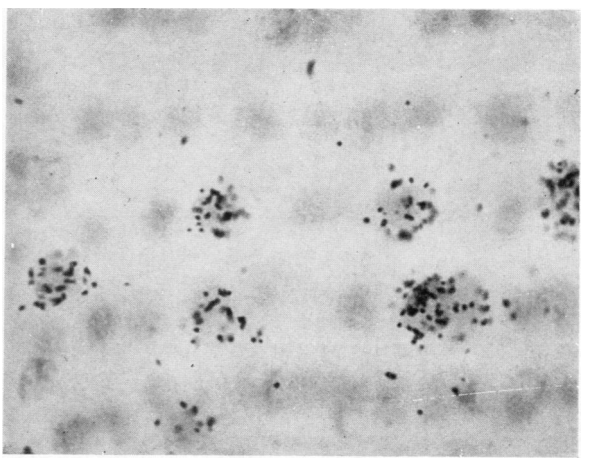

FIG. 1. Autoradiograph of a cross section of *Xanthium* leaf is presented at L.P.I. 0.43. At this developmental stage leaf lamina consists of about 6 layers of meristematic cells. Only 4 layers are included on the photomicrograph. Average diameter of a nucleus is about 5.2 μ. The grains over some nuclei represent relative amounts of ^{3}H-thymidine incorporated into DNA. The leaf was treated for 4 hours with 10 μc/ml of the radioisotope with specific activity 2 c/mmole.

lamina, with higher rates of incorporation and mitotic turnover than any other part of the lamina.

The average number of grains (fig 3) increases as a linear function of concentration of the radioisotopic solution, expressed in μc/ml. A significant fit was achieved for all the lines fitted by least squares method. In all experiments the concentration of 10 μc/ml, and four hours of isotopic growth gave, on the average, about 20 % of labeled nuclei, and 18 grains per average labeled nucleus, which are suitable magnitudes for visual counts and statistical computations.

A 7-fold increase in concentration gives only an increase in grain counts by a factor of 2. It is possible that with an influx of the exogenous ^{3}H-thymidine, some of it is pumped into the vascular system, and is not immediately available for incorporation. A physiologically active plant organ in this case deviates significantly from the theoretically

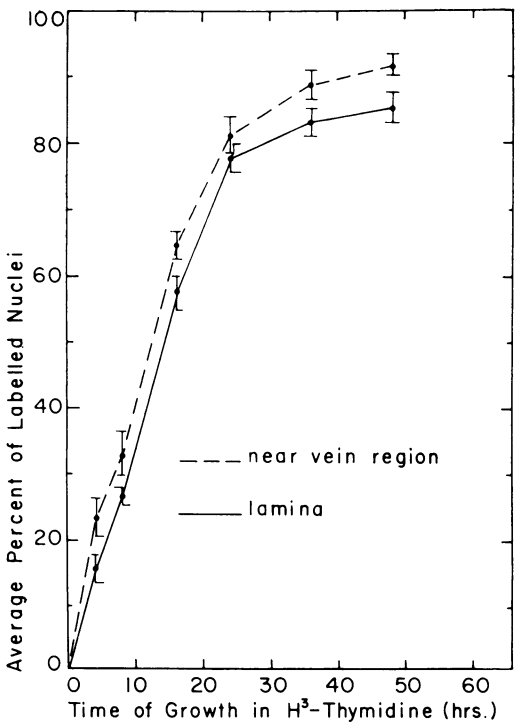

FIG. 2. The average percent of labeled nuclei is plotted vs. time of isotopic growth in hours, for the lamina region near the vein and for the lamina region at some distance from the veins.

expected stoichiometric relationship. Another interesting observation based on figure 3 is the fact that there is no significant difference among the various specific activities as revealed by variance analysis and chi-square tests. This leads to the conclusion that various specific activities of ^{3}H-thymidine (2, 6.7, and 13.7 c/mmole) have no effect upon the amount of the radioisotope incorporated, provided the amount of μc/ml is kept constant.

Figure 4 represents the average number of grains per nucleus on the left ordinate and the rate of

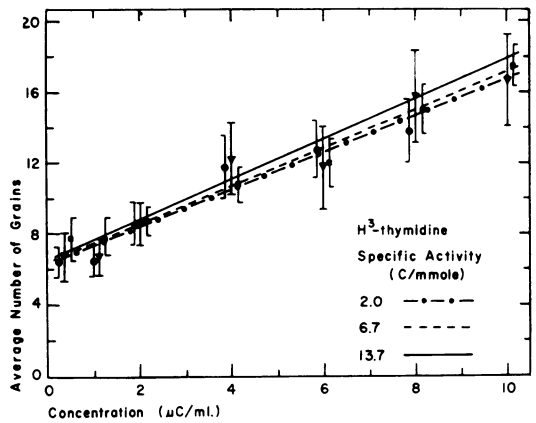

FIG. 3. The average number of grains per nucleus is presented as a function of ^{3}H-thymidine concentration at different specific activities. The various specific activities represented by separate lines on the graph, had no effect upon the amount of the radioisotope incorporation.

incorporation of ^{3}H-thymidine per nucleus on the right ordinate, plotted as a function of the isotopic growth. Since the variance analysis and chi-square tests indicated no significant difference among the various points at 1, 4, and 8 hours of isotopic growth, only one curve was fitted by eye, approximately over the average values of 4 specific activities (2, 3.35, 6.7 and 15.3 c/mmole). The average number of grains per nucleus increases as a logarithmic function of time of growth in ^{3}H-thymidine. These data also support the conclusion mately over the average values of 4 specific activities had no effect upon the amount of tritiated thymidine incorporated. The rate of incorporation (dX/dt) in figure 4 is high during the first hour. and it decelerates with the time of isotopic growth.

Discussion

In the studies on incorporation of radioactive precursors into nuclear DNA, one is confronted with the complexity of this process. An exogenous molecule supplied from the medium has to cross several membrane barriers; it is transported through the cytoplasm and frequently is translocated to distant tissues before being incorporated into nuclear DNA. This, of course, indicates that the process of incorporation is composed of many steps which are still not well understood, and for which no single equation can be proposed at the present time. However, from kinetic studies, one can conclude that the whole process of incorporation, with its component steps, occurs in a controlled and frequently predictable fashion. To mention only a few, it responds with a linear function to external concentrations of the isotope (fig 3) and with a logarithmic function to the time of isotopic growth (fig 4).

There are several points which should be discussed in relation to figure 2. It should be emphasized that the slopes of the lines representing the

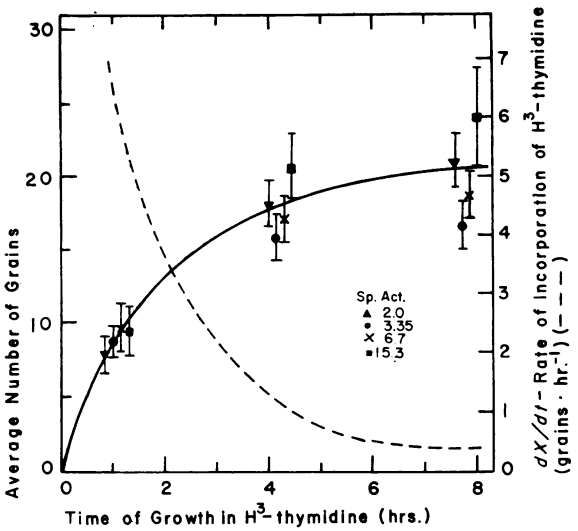

FIG. 4. The average number of grains per nucleus and the rate of ^{3}H-thymidine incorporation are plotted vs. time of isotopic growth. The solid-line curve approximates a logarithmic function. Rates of incorporation are high during the first hour, rapidly decelerating thereafter.

average percent of labeled nuclei would change depending on the established criteria as to what minimal number of grains should constitute a specifically labeled nucleus. The cell population of the lamina with 4 hours of isotopic growth has a high frequency of nuclei with 1, 2, 3, 4 and 5 grains (11). To satisfy the $P<0.01$ level of significance with respect to the background radiation, it was necessary to consider nuclei as labeled only if they were associated with 6 or more grains. For $P<0.05$ level of significance it would be necessary to use 5 grains over 1 nucleus. If one would plot the number of labeled nuclei with 5 grains or less versus the time of isotopic growth, the slopes of the curves would become steeper depending upon the selected criteria. A significant proportion of labeled nuclei with 5 grains or less was rejected in figure 2 to assure 99 % certainty that the assessed nuclei were specifically labeled. The percent of labeled nuclei increases as a function of growth in ^{3}H-thymidine. The duration of the isotopic growth will affect the proportion of the cells going through the S period. Since the average generation time for *Xanthium* cells is about 22 hours with an S period of about 15.7 hours (6) one would expect that after about 22 hours all cells that went through that period should appear as labeled. However, a 100 % level of labeled nuclei in figure 2 was not reached. One of the possibilities is that not all cells are undergoing division in terms of DNA synthesis during a period of 48 hours. Some of these cells may be in a process of differentiation or have already differentiated into specialized tissues. This conclusion would be in agreement with the cellular morphology of lamina development (10) in the *Xanthium* leaf.

Quantitative autoradiography usually represents relative measurements (14), but from grain density data, if autoradiographic efficiency is known, it is possible to calculate the absolute amount of the isotope incorporated. The magnitude of error will depend primarily on the autoradiographic efficiency. Calculations based on the assumption that 20 disintegrations represent 1 grain (1, 2, 15) reveal that 1.1×10^{-8} μc of the radioisotope was incorporated per 74 μ^3 of the average nucleus at 4 hours of isotopic growth. An attempt was also made to estimate the number of molecules incorporated into DNA per 74 μ^3 of the average labeled nucleus (table II). After 4 hours of isotopic growth, with a concentration of 10 μc/ml and specific activity of 2 c/mmole, a nucleus has incorporated a total

Table II. *Incorporation of Thymidine and ^{3}H-Thymidine Molecules into Nuclear DNA*

Isotopic growth	Sp act	Total* no of molecules·10^5	dX/dt-Rate of incorporation*	No of ^{3}H-thymidine molecules·10^5**	dX/dt-Rate of incorporation**
hrs	c/mole		No of molecules·10^5·hr^{-1}		No of tritiated thymidine molecules·10^5·hr^{-1}
1	2.00	17.80	12.40	1.201	0.850
	3.35	10.63	7.00		
	6.70	5.31	3.60		
	15.30	2.33	1.60		
4	2.00	33.45	2.85	2.256	0.190
	3.35	19.97	1.75		
	6.70	9.98	1.10		
	15.30	4.33	0.27		
8	2.00	39.51	0.70	2.665	0.055
	3.35	23.56	0.40		
	6.70	11.79	0.15		
	15.30	5.16	0.12		

* Labeled and unlabeled.

** The number of ^{3}H-thymidine molecules and their rates of incorporation are approximately the same for each specific activity.

of 3.34×10^6 thymidine molecules, of which 2.25×10^5 were labeled. Assuming that 1 tritium atom is associated with 1 thymidine molecule, and that no isotope effect is operative in the system, approximate ratios of ^{3}H-thymidine to thymidine molecules can be estimated. Specific activities of 2, 3.35, 6.7 and 15.3 c/mmole give ratios of about 1:14, 1:8, 1:3 and 1:1 of labeled to unlabeled molecules respectively. The number of tritiated molecules incorporated into nuclear DNA remains constant for any specific activity. Changes in the specific activity affect only the number of unlabeled molecules and also the molar concentration of the incubation solution. Increased molar concentrations result in higher rates of incorporation of the total number of molecules. It is believed that the differential rates of uptake and incorporation are responsible for the situation, where in 1 case, packages of 1:14, and in the other 1:1 packages of the labeled to unlabeled molecules are incorporated. This would be possible in an unsaturated system, in which the exogenous supply of molecules is smaller than the endogenous thymidine pool. It is hypothesized that under a saturated condition one would expect a decrease of ^{3}H-thymidine incorporation, expressed by a drop in the number of grains per average labeled nucleus. A cell cannot distinguish between tritiated and non-tritiated moieties. Increase in the number of carrier molecules should result in a progressively smaller uptake of the labeled molecules since the probability law of random selection should be applicable in this system.

Cleaver and Holford (2) studied ^{3}H-thymidine incorporation into DNA of mouse L-strain cells. They were interested in the exogenous and endogenous thymidine pools involved in DNA synthesis. This investigation is limited to the kinetics of incorporation. However, some parallels can be discussed between the 2 studies. They concluded that ^{3}H-thymidine was incorporated continuously into DNA at a constant rate up to 1 hour at concentrations between 0.01 and 40 μM. At concentrations of the external medium higher than 2 μM, the incorporation mechanism appeared to be saturated and the incorporation rate was independent of the concentration of thymidine in the external medium. Considering the average number of grains per nucleus, the exposure time and the time of isotopic growth, the leaf cells display significantly smaller rates of incorporation. Perhaps because of the slugishness of the system, a saturation level in leaf nuclei was not reached with 5 μM thymidine concentration and 4 hours of incubation. This would indicate that both different physical barriers and physiological mechanisms may be operative in the 2 systems, with similar kinetic patterns but different magnitude of rates.

One should realize that not only are there differences between animal and plant tissues, but there also are differences among various plant organs such as roots and leaves. As evident from the work of Wimber and Quastler (16, 17) root cells can be labeled efficiently with significantly lower concentrations of ^{3}H-thymidine and a shorter period of isotopic growth than the *Xanthium* leaf cells.

It is believed that the observations reported in this paper will be applicable in physiological studies of roots, stem apices and leaf development with application of tritium labeled precursors. They also may prove valuable in investigations dealing with nucleic acid metabolism of these plant organs.

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