ANALYSIS OF TRITIUM INCORPORATION INTO INDIVIDUAL CELLS BY AUTORADIOGRAPHY OF SQUASH PREPARATIONS

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ABSTRACT

The relation between tritium content of individual cells and grain count obtained in autoradiographs of squashed cells was investigated. The tissues used were root meristems of *Tradescantia paludosa* and intestinal epithelium of the mouse. The relation between grain count and tritium content is affected by self-absorption which depends on the thickness of the labeled cell. Therefore, squashed preparations were sectioned to determine the uniformity of thickness of nuclei. In a preparation of mouse cells, thicknesses were $1.18 \pm 0.35\ \mu$, and in a preparation of *Tradescantia* cells, $2.97 \pm 0.35\ \mu$. The effects of similar and larger variations in thickness upon grain count were studied in material squashed with different pressures; no marked correlation was found. The lack of correlation is explained by the geometric relation between labeled nuclei and the emulsion. By counting grains and directly measuring tritium content in a glass proportional counting tube in the same preparation, the yield of grains per disintegration was measured in *Tradescantia* cells and found to be 1 grain for 10.9 disintegrations with AR 10 autoradiographic film and 1 grain for 19.3 disintegrations for NTB nuclear track liquid emulsion. Latent image fading may pose a problem with long exposures; the conditions of its occurrence are as yet not well known.

INTRODUCTION

Tritium has a half-life of 12.8 years and emits beta rays that travel in tissue from less than one micron to a few microns (1). It can be stably incorporated into several organic substances, *e.g.* nucleosides and amino acids, that are utilized by cells in the synthesis of biological macromolecules. For these reasons, tritiated compounds are very useful in high resolution autoradiography. The tritium-labeled compound which has been used most for autoradiography is thymidine which goes preferentially into deoxyribonucleic acid (DNA). It has been used to determine the loci of DNA synthesis, to follow the movements of cells, and to estimate rates of DNA synthesis.

The number of silver grains activated over a cell is related to the number of disintegrations

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which have occurred in that cell during exposure of the emulsion. Therefore, the grain count over a given cell can be used as an index of the amount of H^3-thymidine incorporated in that cell. This relationship, however, is complicated by self-absorption; a tissue section of conventional thickness, *viz.* 5 to 15 μ, is much thicker than the range of most beta rays from tritium. Thus, the degree to which a particular labeled nucleus will register depends on its distance from the surface of the section. Most of the grains activated will be due to tritium within the topmost half-micron of tissue. Under these circumstances, the mean grain count per cell (or per labeled cell) and the maximum grain count per cell are meaningful; but a detailed distribution of grain counts has little value. On the other hand, the grain count over individual cells in very thin sections (1 μ or less) is a fair approximation of the intranuclear concentration of tritium. However, such ultrathin sections are not easily produced. The method of choice for the study of the tritium content of individual cells is the squash technique with Feulgen staining; the intercellular matrix and much of the cytoplasm is removed, and the nuclei are reduced to a fairly uniform thinness which minimizes variation due to self-absorption.

This study deals with the relation between tritium content of individual cells and grain count as obtained in autoradiographs of squashes. Since the thickness of the cells may be of critical importance, the range of cell thicknesses in a given preparation was studied by the most direct method, *viz.* sectioning squashed material. Next, similar tissues were squashed with various pressures and the relation between mean grain count and degree of flattening was investigated. Finally, the efficiency of intranuclear tritium disintegrations in activating photographic silver grains was measured.

RESULTS AND DISCUSSION

Squash Sections

Squashes are prepared by flattening cells between two rigid plane surfaces; therefore cells in a given preparation must have similar thicknesses. To obtain precise information on the degree of uniformity, sections were prepared from squashed material. The epithelium of the intestinal crypts of the mouse as well as root tips of *Tradescantia paludosa* were used in the study. Tissues were fixed in ethanol-acetic acid (3:1), stained by the Feulgen method, and squashed between a slide and coverslip which had been previously covered with thin coatings of celloidin. The

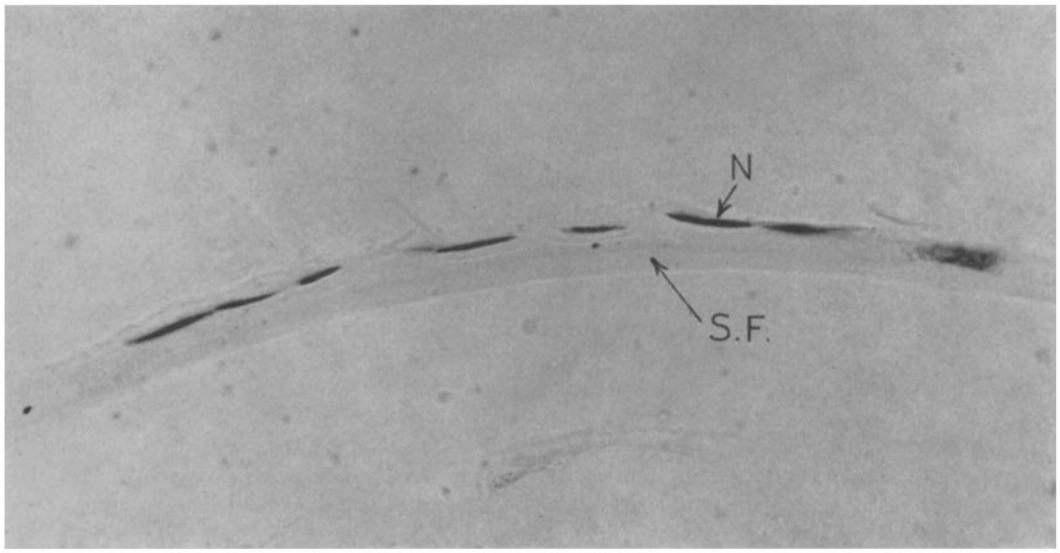

FIGURE 1

Section of squashed intestinal epithelium. The plane of the section is perpendicular to the plane of the squash. Nuclei (*N*) are stained. Stripping film (*S.F.*). × 900.

coverslips were removed by the dry ice method (2). The celloidin on the coverslip nearly always remained with the tissue on the slide which thus was sandwiched between two layers of celloidin. The slide was placed into absolute ethanol and passed through the alcohol series into water. At this stage, stripping film was placed over the tissue to facilitate handling. The stripped slides were carried through the tertiary-butyl alcohol series (3) and the preparation (tissue plus stripping film) peeled from the slide and embedded in paraffin. Sections were made at 10 μ in the direction perpendicular to the plane of the slide.

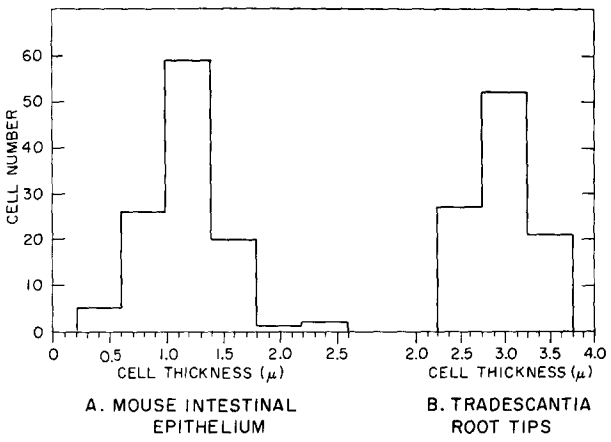

FIGURE 2

Frequency distribution of the thickness of nuclei measured from squash preparations that were sectioned in a plane perpendicular to the squash.

Fig. 1 shows a section through a squashed preparation of intestinal epithelium. Sections of squashed nuclei appear as fairly regular rectangles of approximately even thickness. Actual measurements of thickness obtained on large numbers of cells are shown in Fig. 2. In the particular preparations used, intestinal cells averaged 1.18 μ in thickness, with a standard deviation of 0.35 μ, that is 29 per cent of the mean; *Tradescantia* cells averaged 2.97 μ with a standard deviation of 0.35 μ or 12 per cent of the mean.

Degree of Flattening and Grain Count

The study of sectioned squash preparations has shown that even squashed cells are thick enough for considerable self-absorption, and that the variations in thickness may be large enough to cause significant differences. To test the effect of degree of flattening upon the grain count, a series of squashes was prepared under purposely varied pressures. Several mice were used in this study and from each one several slides were prepared of closely neighboring portions of the intestinal epithelium. Labeling was done by a single intraperitoneal injection of H^3-thymidine about ½ hour before sacrifice. The slides were dipped in Kodak NTB nuclear track liquid emulsion and stored in light tight boxes at 4°C. They were processed (after exposure) in Kodak D-19 for 6 minutes and transferred to acid fixer. The degree of flattening was estimated indirectly. For a given nuclear volume, the product of the thickness of a squashed nucleus times its optical cross-section (area as seen from above) must be constant. The relation between grain count and mean optical cross-section for 30 crypts so studied is shown in Fig. 3. The degree of flattening (*i.e.*, mean thickness) varied by more than threefold; yet the mean grain counts, though showing some variation between slides from the same animal, were not strongly correlated with the thickness of the nuclei.

The reasons for this lack of strong correlation may be as follows. Most of the autoradiographic effect of intranuclear tritium should be due to a surface layer of about ½ μ thickness. The fraction of the volume comprised in this layer can be roughly equated to the autoradiographically effective fraction of the total volume. A squashed

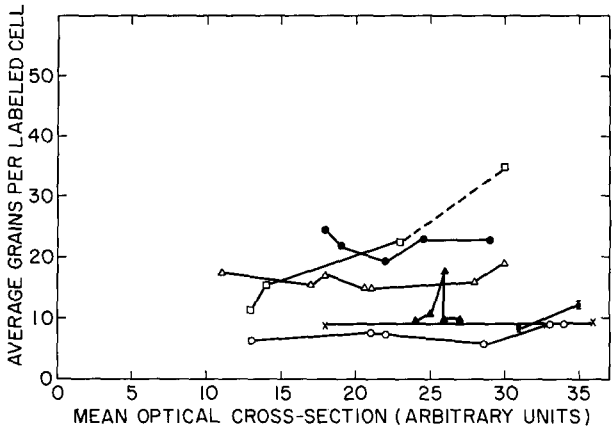

FIGURE 3

Correlation of the optical cross-section (degree of flattening) of nuclei with the average number of activated silver grains per nucleus. Each type of symbol represents crypt squashes taken from the same general area of the intestinal epithelium of one mouse.

nucleus has approximately a cylindrical shape. Since the photographic emulsion, particularly with the dipping technique, is apposed to both the sides and the top of the cylinder, the active layer includes both of these surfaces. The harder a cell is squashed, the greater the area of the top but also the smaller the height of the side walls. These two effects tend to compensate each other for the heights of squashed nuclei commonly observed (see Fig. 1) as shown in Table I.

Since twofold differences in the mean degree of flattening of cells result in only 30 to 50 per cent differences in expected mean grain count, it may be concluded that the much smaller variations in individual degree of flattening within a given squash preparation have no significant effect upon the individual grain count. (This is true for preparations dipped in NTB liquid emulsion; it may not apply to stripped preparations in which the sides of the nuclei are not so intimately associated with the emulsion.)

Radiographic Efficiency

The yield in photographic grains per disintegration was found as follows. Rooted plantlets of *Tradescantia paludosa* were grown for 24 hours in nutrient solution to which 2 μc/ml. H^3-thymidine had been added. Two root tip squashes were prepared: one was stripped with Kodak AR 10 fine grain autoradiographic film and exposed for 6 hours while the other was dipped into Kodak NTB nuclear track liquid emulsion and exposed for 19 hours before processing. On each slide the number of grains over a random sample of 500 intact cells was counted and the total number of cells per slide was also determined. On this basis, the total number of grains on the entire slide was estimated. Subsequently, the material on each slide was removed in concentrated nitric acid and the radioactivity of an aliquot part of the solution determined in a glass proportional counting tube (4). The sample was combusted in a sealed tube over nickel oxide and zinc. The combustion products were introduced into the counting tube along with a filling gas of 90 per cent argon–10 per cent methane.

The counting efficiency was found to be about 98 per cent. For the AR 10 stripping film there were about 10.9 disintegrations per grain or about

TABLE I

Autoradiographically Effective Fraction of Nuclear Volume vs. Height of Squashed Nucleus

Height of squashed nucleus, μ	Effective fraction
(*a*) Intestinal epithelium mouse (mean nuclear volume 130 μ^3)	
0.8	0.67
1.2	0.51
1.6	0.44
2.4	0.39
(*b*) *Tradescantia* roots (mean nuclear volume 700 μ^3)	
2.0	0.31
2.5	0.29
3.0	0.27
3.5	0.25
4.0	0.24

TABLE II

Autoradiographic Yield

Emulsion	Total cells on slide	Mean grains/ Cell	Estimated Total grains/Slide	Disintegrations/ Exposure period	Disintegrations/ Grain	Estimated efficiency
						per cent
AR 10	26,400	26.2	691,800	7.56×10^6	10.9	9
NTB	32,750	26.7	874,425	16.92×10^6	19.3	5

a 9 per cent efficiency. With the NTB liquid emulsion, there were 19.3 disintegrations per grain, or about 5 per cent efficiency. A summary of these findings is given in Table II.

Since the size of the flattened cells is large compared to the range of the tritium beta particles, the radiographic efficiency will decrease with an increase in size of the structure tested (though, as we have seen, not much with the degree of flattening when the NTB liquid emulsion is used). The intestinal cells are considerably smaller than the *Tradescantia* cells and should give about twice the autoradiographic yield. Thus, with liquid emulsion, the yield should be about 10 per cent.

Because tritium has a half-life of 12.8 years, the total number of electrons stopped by the emulsion increases practically linearly for exposure periods up to a year. In some tests, the grain count was found to increase accordingly. For instance: two mice were given 5 μc. of tritiated thymidine and several preparations of the intestinal epithelium made of each. The slides were exposed for 2, 8, 33, and 64 days under three conditions: at 4°C. with and without drierite (anhydrous calcium sulfate), and at −17°C. with drierite. No significant difference between treatments was found, and the average grain counts over the nuclei showed roughly a linear increase with time. However, the grain count does not always increase. Several factors, at least some of which are poorly defined, can cause fading of a latent photographic image (5). We have never noticed such an effect with exposure times up to two weeks, but with exposure times of 1 or 2 months we have sometimes found considerable fading.

CONCLUSIONS

The detailed autoradiographic evaluation of squash preparations yields a fairly precise estimate on the distribution of amounts of tritium incorporated. This, in general, is proportional to the amount of exogenous thymidine incorporated during the labeling period. With short labeling periods, the amounts incorporated are proportional to instantaneous rates of incorporation. These in turn depend on the rate of DNA synthesis; a number of factors complicate the relation between rate of thymidine incorporation and rate of DNA synthesis such as differences in pool size, in membrane permeabilities, in relative blood flow, etc. If these factors are duly taken into account, then the autoradiographic study of squashes can yield information on distribution of instantaneous rates of DNA synthesis—that is, the information complementary to that on total amounts of DNA which is furnished by microphotometric methods.

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