THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 57, No. 6

Printed in U.S.A.

FOLLICLE SQUASH PREPARATIONS: USES IN STUDIES OF CELL KINETICS FOLLOWING IRRADIATION*

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ABSTRACT

These investigations in CF_1 mice were undertaken to establish the usefulness of whole follicle squash preparations in kinetics studies of anagen matrix cell responses to ionizing radiation. The backs of mice were plucked and H^3TdR (0.8 uCi/g) was injected intraperitoneally 15 minutes prior to irradiation. Animals were treated with 100 rads at 45 kv. Skin biopsies were fixed in cold acid alcohol for 24 hours, brought through graded alcohols to water, hydrolyzed in 1N HCl, and transferred to Schiff's reagent overnight. Individual follicles were dissected free and squashed on slides in 45% acetic acid. Autoradiographs were made from squash preparations.

Mitotic indices were reduced within 30 minutes after x-ray exposure (2.4% in control sites vs 1.8% in irradiated sites) and dropped sharply after 2 hours (2.2% vs 0.7%). There was essentially complete recovery of the mitotic index within 7 hours after irradiation (2.1% vs. 1.7%). The post-radiation G_2 period delay was 1½–2 hours. Studies of the 4 individual hair types revealed no significant differences in radiation responses despite substantial differences in the sizes of matrix cell populations. Usefulness of the technique described for kinetics studies of drug or radiation effects on proliferative cell populations is emphasized.

Hair matrix cell populations are unique: periods of exceedingly active cell reproduction alternate regularly with periods of complete reproductive inactivity. The hair matrix thereby provides a useful indicator system for in vivo studies on the effects of ionizing radiation or a wide variety of pharmacologic agents on rapidly dividing or non-proliferating cell populations (1). The current experiments were undertaken as an initial study of matrix cell kinetics following a single low dose of ionizing radiation.

Although mouse skin sections obtained for this study were satisfactory, limitations of the standard techniques for fixation and sectioning included problems of tissue alignment, need for examination of large numbers of follicles, and the inabilty to identify follicles according to hair type. To overcome these difficulties and to enjoy the advantage of working with whole, discrete matrix cell populations we modified and adapted earlier tissue techniques (2) to the dissection for study of single hair follicles obtained from skin biopsy specimens (3, 4).

Received June 18, 1971; accepted for publication August 10, 1971.

This investigation was supported by a grant from the Environmental Control Administration, CPEHS, U.S. Public Health Service, EC00084-08; General Research Support Grant RR05477 from the General Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health; and by the United States Atomic Energy Commission.

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MATERIALS AND METHODS

All studies were carried out with Carworth Farms No. 1 female mice, three months of age. Hair was plucked from a 7 square centimeter area on the back above each thigh in all animals to induce anagen, which lasts about 19 days in this strain of mouse. Fourteen days later all animals received an intraperitoneal injection of 0.8 microcuries of tritiated thymidine (H^3TdR) per gram body weight (specific activity 14.0 Ci/mmole). Radiation was given 15 minutes later. Skin biopsies were taken at multiple time intervals between 30 minutes and 5 days after irradiation. All biopsy specimens were taken with a 3 mm dermal punch without anesthesia. A maximum of 6 biopsies each from control and treatment sites was taken from any one animal. Data for each post-treatment interval were obtained from 1 or 2 test animals.

Radiation was delivered with a Machlette OEG 60 tube operated at 50 kv and 30 mA with 2 mm

Al added filtration. This produced a surface dose of 402 rads per minute at a focal distance of 11-cm, and a beam quality of 1.2 cm half-value depth in tissue. A single 2 cm portal was irradiated on the right back above the thigh. All animals received a single surface dose of 100 rads. The animals were adequately shielded and treated individually with intraperitoneal nembutal anesthesia.

All biopsy specimens were immediately fixed in cold acid alcohol (1 part glacial acetic acid and 3 parts absolute alcohol) for 24 hours. The specimens were then transferred to 70% ethyl alcohol and brought through a graded series of alcohols to water. Each tissue sample was then hydrolyzed in 1N HCl at 60°C. for 10 minutes. Hydrolysis was stopped by placing the samples in cold distilled water. The biopsy specimens were next immersed in Schiff's reagent for the Feulgen staining reaction, and kept in the dark at room temperature overnight. Small pieces of biopsy specimens were then placed in a drop of 45% acetic acid on a standard microscope slide. Individual follicles were dissected out under a dissection microscope, with care being taken to identify each follicle according to hair type. One to six follicles were placed on a slide, covered, and gently "squashed" by firm overhead pressure applied to the coverslip (Fig. 1A, 1B). The coverslip was then "popped" off after brief exposure of the slide to dry ice. The slide was thawed in fixative, air dried, and prepared for autoradiography.

Slides were dipped in Ilford K_2 Nuclear Emulsion and exposed for three to four weeks in a dry atmosphere at 4°C. The slides were developed in Kodak D 19 developer. Exposure time was generally regulated to yield mean grain counts in a range of 20 to 25 grains.

In these experiments a minimum of 12,000 cells were scored to obtain the mitotic and labeling indices for each control and radiation site. Approximately ten hair matrices were studied for each control and radiation point. A minimum of 150 mitotic figures was counted to obtain the percent labeled mitoses for both irradiated and control sites. Since background in the autoradiographs was low, labeled cells were defined as cells containing three or more grains.

RESULTS

The results of this study are shown in Figures 2–4. Within 30 minutes after exposure of anagen

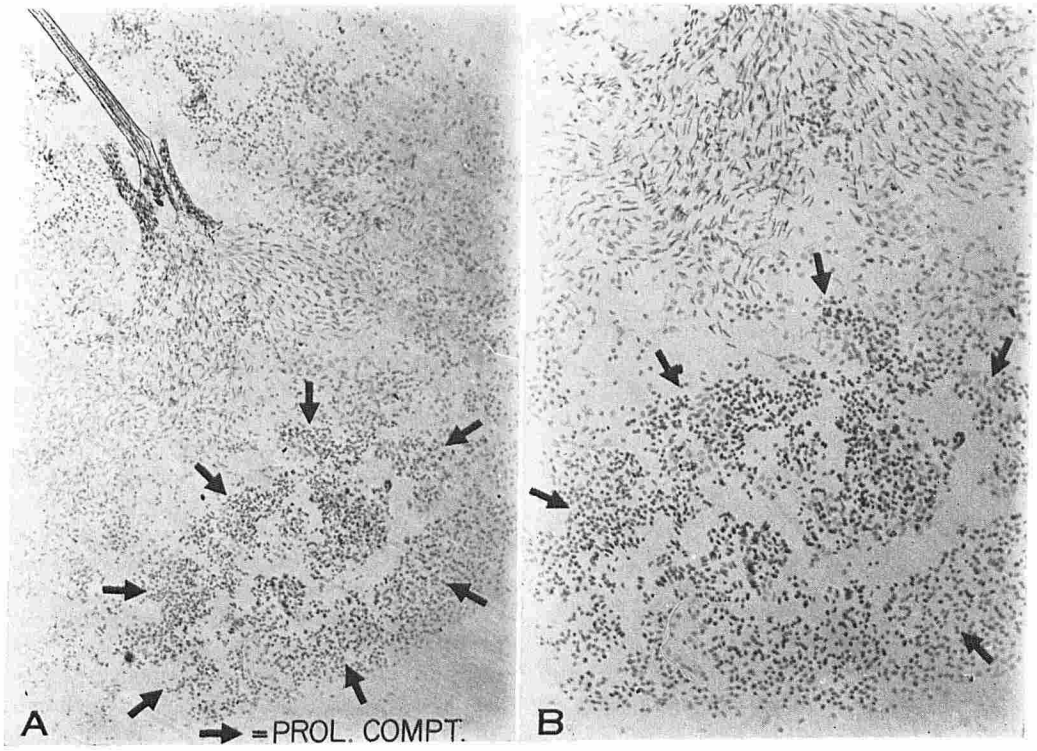

FIG. 1A. Squash of awl hair (× 50). Hyperchromatic nuclei in lower part of photomicrograph comprise the proliferative cell compartment (Prol. Compt.), circumscribed by arrows.
FIG. 1B. Magnification (× 79) of awl hair from Figure 1 showing differentiating cells migrating toward the hair shaft, and proliferating cells below (circumscribed by arrows).

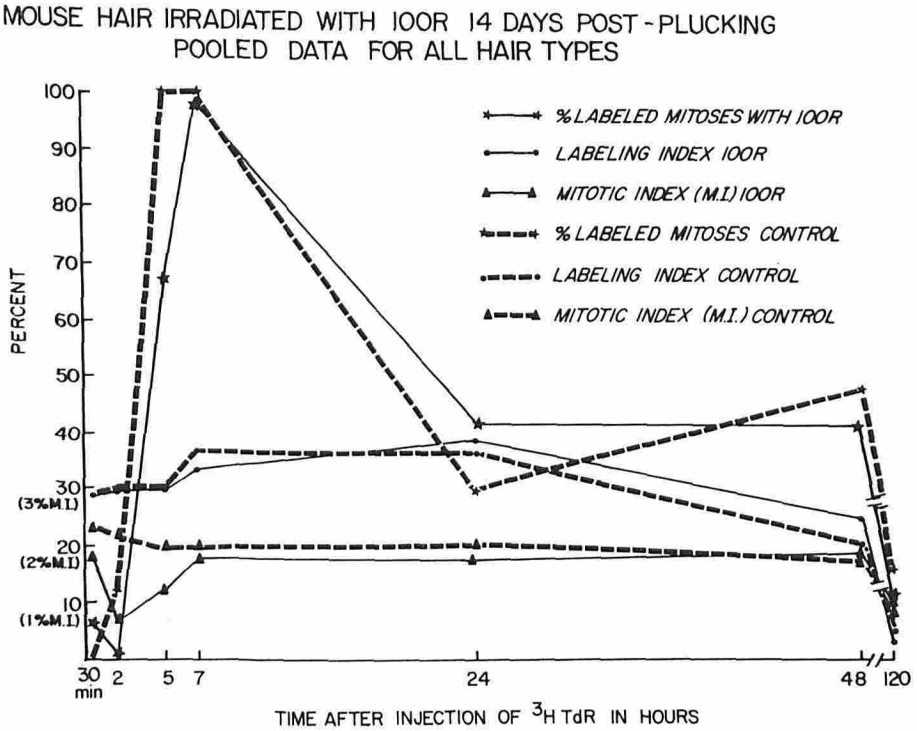

FIG. 2.

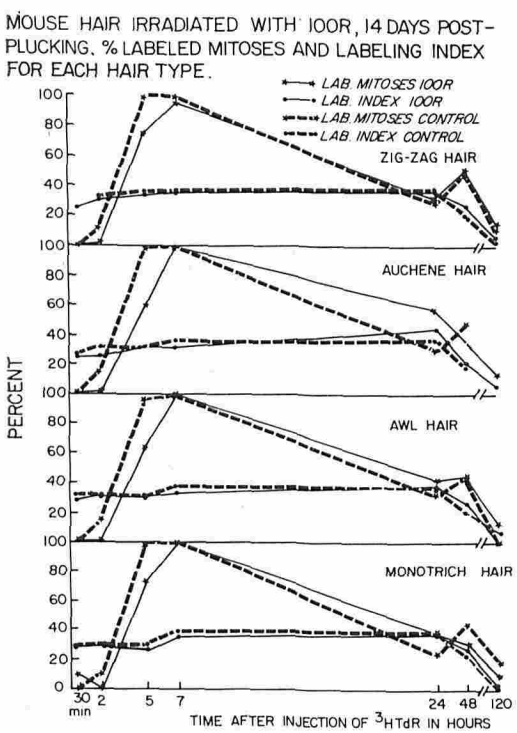

FIG. 3.

hair matrices to single radiation doses of 100 rads, the mitotic index was reduced to 1.8%, compared with 2.4% in the control sites. A further sharp drop in the mitotic index to 0.7% was found 2 hours post-radiation. At this 2 hour interval labeled mitoses, present in unirradiated follicles, were absent in irradiated areas, indicating a post-radiation G_2 delay in the mitotic cycle. At 5 hours, the next post-radiation time interval studied, 67% of mitoses found in the irradiated areas were tritium labeled, reflecting a substantial release of cells from the radiation-induced division delay. Since the G_2 period in the proliferative cell population lasts 2 to 2½ hours (3), the increase in labeled mitoses from zero (2 hour interval) to 67% (5 hour interval) three hours later, suggests release of a block induced largely in G_2 cells. Termination of the radiation-induced block was accompanied by a significant partial recovery of the mitotic index to 1.3%. At 7 hours post-radiation the mitotic index in irradiated sites was 1.7%, suggesting essentially complete recovery. At this same time interval all mitoses were tritium labeled. Comparison between the percent labeled mitoses curves of irradiated areas and of either control

sites in this study or control sites sampled at hourly intervals in normal follicles studied previously (3), indicated a total division delay (largely G_2 period) of 1½ to 2 hours.

At twenty-four and forty-eight hours after irradiation no significant further changes were observed in the mitotic index. The return to a normal cell production pattern indicated that most damaged cells had either recovered or had been eliminated from the proliferative compartment. No temporary increase or "overshoot" phenomenon in the mitotic index was found in irradiated follicles following the recovery phase, but more frequent tissue sampling would be needed to study this question adequately.

The reduction in labeling index appearing in both radiation and control sites at the 48 hour interval (Figures 2, 3) reflects two factors: 1) that labeled cells had passed through at least three division cycles following administration of tritium labeled thymidine, thereby diluting the label (generation cell cycle time in normal mouse hair matrix cells is about 13 hours [3]); and 2) many labeled cells had been eliminated from the proliferative compartment.

The sharp reduction in the mitotic and labeling indices (Figures 2–4) in both control and irradiated sites at 120 hours was to be expected since, at that time interval, all hairs were in the 19th day of anagen. Our previous studies have shown that the number of hair matrix cells in DNA synthesis and in mitosis drops precipitously from normally high levels to zero between days 18 and 20 of anagen, as the hair growth cycle terminates abruptly (3).

Radiation-induced cellular damage was reflected in the appearance of some pyknotic cells (Figure 5), and abnormal mitoses (Figure 6). Pyknotic cells, first visible 5 hours post-radiation, were most numerous at the 7 hour interval, comprising 7–8% of cells in the proliferating cell compartment. Both labeled and unlabeled pyknotic cells were found. Dividing pyknotic cells were extremely rare, and pyknotic cell mitoses, when seen, were often abnormal. These damaged cells were greatly decreased in number 24 hours post-irradiation, and were not found in the follicles 48 and 120 hours after radiation.

The findings discussed above comprise composite data for the 4 principal hair types present in the mouse's body coat. In the anatomical sites (flanks) studied, overall distribution among the major hair types was as follows:

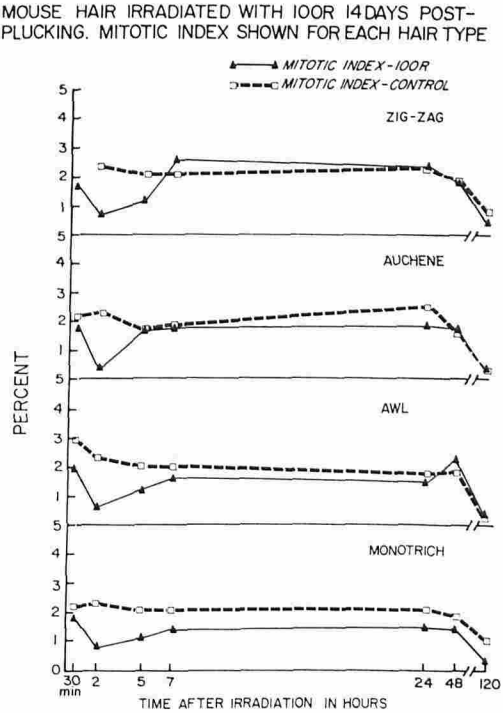

FIG. 4.

* Monotrich (Type A)	1.7 %
Short monotrich (Type A–B)	1.1 %
Awl (Type B)	20.0 %
Auchene (Type C)	11.6 %
Zig-Zag (Type D)	65.6 %

Data present in Figures 3 and 4 reveal that no significant differences in radiation responses were observed among the four major hair types.

In general, follicle squash preparations showed that proliferative cell compartments in monotrich hair matrices normally contain up to twice the cell populations found in matrices of other hair types (see Table). (Proliferative compartments were identified and characterized in this study by well demarcated collections of cells with identical morphologic characteristics and tinctorial affinities. See areas circumscribed by arrows, Figures 1A and 1B.) Determinations of mean labeling indices and mean mitoses per follicle reveal that, in proportion to the total cell populations, these parameters are similar for all four hair types (Table).

* Dry's classification of hairs. From: Dry, F. W.: The coat of the mouse (Mus musculus). Jour. Genet., *16:* 287, 1926.

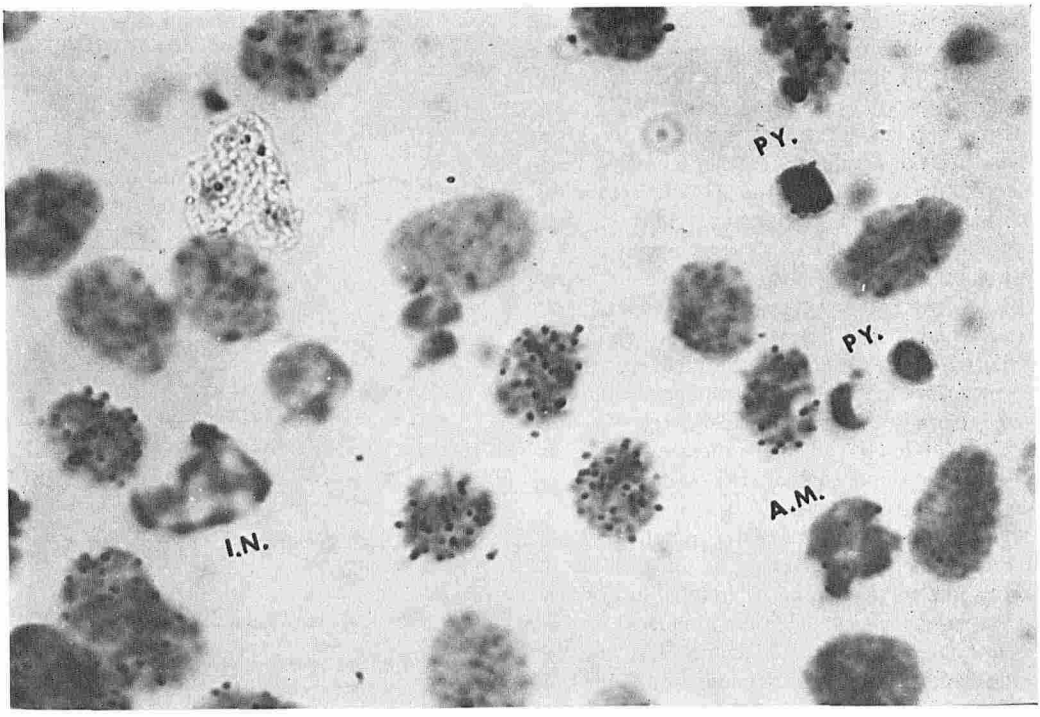

FIG. 5. Pyknotic nuclei (Py), abnormal mitosis (A.M.), and radiation-damaged interphase nucleus (I.N.) can be seen. × 1250.

DISCUSSION

Although cell proliferation rates are extremely high in such tissues as hair matrices, intestinal crypts, and bone marrow, the rate is sensitive to numerous factors including physical injury, altered nutritional status, vascular change, various pharmacologic agents, ionizing radiation, etc. Exposure of proliferating cell populations to ionizing radiation reduces cellular mitosis by inducing a delay prior to cell division (5, 6). In most cell lines the severity and duration of this effect are linear with dose (7). Although division delay occurs in cells irradiated at any stage of the proliferative cycle, the magnitude of this effect is directly related to cell age or position in the cell cycle. Division delay results primarily from a temporary radiation-induced G_2 block which occurs in cells irradiated at all stages of the cell cycle (8). In addition radiation also produces a G_1 delay prior to the onset of DNA synthesis (or S phase) and a prolongation of the S phase in most cell types (7, 9–11). Studies of different cell lines have provided evidence that impaired protein synthesis is the principal factor accounting for post-radiation delay in progression of the cell cycle (12–14). Recent studies by Bacchetti and Sinclair have linked this delay to a requirement for excess protein synthesis needed to repair cell damage (15). Cellular target sites for radiation-induced mitotic delay appear to be nuclear or to reside in the immediately contiguous perinuclear cytoplasm (16).

Data gathered from the current study reveal that hair matrix cells show a substantial reduction in cell proliferation rates even after relatively low doses of ionizing radiation. Division delay is approximately one minute per rad following exposure to 100 rads. Recovery to normal cell division rates is rapid and essentially complete 7 hours later. The appearance of some pyknotic cells as early as 5 hours post-radiation, however, indicates that a fraction of matrix cells (less than 10%) fails to undergo further sustained cell division during the remainder of anagen. Studies to determine whether radiation effects on the mitotic index and division delay are linear with dose are currently in progress.

The follicle squash preparations used in this

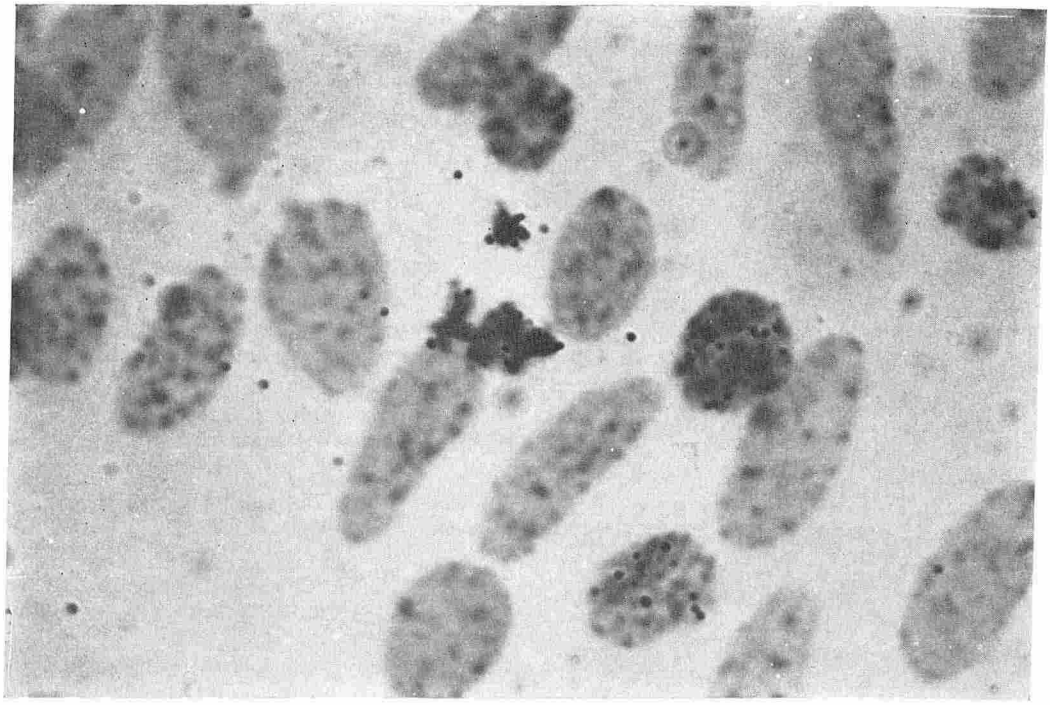

FIG. 6. Autoradiograph showing abnormal fragmented mitotic nucleus 24 hours after exposure to 100 rads. × 1250.

TABLE

Proliferative cell populations and reproductive activity (labeled cells and mitoses) in normal 14 day follicles and in 14 day follicles irradiated with 100 rads. Combined data are shown for each hair type comprising 30 minute, 2 hour, 5 hour, and 7 hour points. Tritiated thymidine was administered 15 minutes before irradiation. Mean numbers and standard deviations (S.D.) are shown.

Hair types	Normal follicles			Irradiated follicles (100 rads)		
	Cells in proliferative cell compartment	Labeled cells	Mitoses	Cells in proliferative cell compartment	Labeled cells	Mitoses
	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.	Mean S.D.
Monotrich	1739 ± 282	568 ± 166	37 ± 6	1708 ± 289	517 ± 102	22 ± 10
Awl	1183 ± 226	358 ± 44	25 ± 5	1188 ± 159	369 ± 47	16 ± 6
Auchene	874 ± 102	258 ± 48	19 ± 6	842 ± 97	249 ± 47	13 ± 5
Zig-zag	788 ± 42	261 ± 26	17 ± 4	742 ± 93	232 ± 40	10 ± 7

study present several advantages over the use of skin sections, as alluded to earlier. They are particularly useful in permitting determinations of total cell populations in matrix cell proliferative compartments, as well as accurate assessments of the total number of cells undergoing mitosis or labeled with tritiated thymidine. The squash preparations provide a fairly precise evaluation of all parameters of cell kinetics within a finite and measurable cell population, as well as providing a useful technique for the study of cell maturation and loss from the hair matrix. Nonetheless, in some follicles, even after prolonged study, it is not always possible

to delineate the precise boundaries of the proliferating cell compartment within the matric cell population.

The dissection of single whole follicles also provides a technique for combustion and assay of radio-activity in single follicles or in small groups of follicles with tritium labeled matrix cell populations. This method permits close correlation of uptake measurements with autoradiographic findings (4).

Hair matrix cell populations, with their alternating cycles of rapid proliferative activity and complete reproductive inactivity, provide a suitable model for the study of factors controlling cell division and growth. This easily accessible cell system also presents us with an unusual opportunity to study effects of radiation or pharmacologic agents on cell kinetics and survival. Such observations may also provide information on suitable therapeutic approaches, utilizing single or combined agents, for the treatment of benign and malignant disorders characterized by basic abnormalities in cellular proliferation or differentiation. Perhaps the techniques and findings described in the current study can be extended to encompass some of these goals.

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