[5] W. A. Bulen and D. S. Frear, *Arch. Biochem. Biophys.*, **66**, 502–503, 1957.
[6] P. W. Wilson and S. G. Knight, *Experiments in Bacterial Physiology* (Minneapolis, Minn.: Burgess Publishing Co., 1952).
[7] D. Burk and R. H. Burris, *Ann. Rev. Biochem.*, **10**, 587–618, 1941.
[8] R. W. Stone and P. W. Wilson, *J. Biol. Chem.*, **196**, 221–225, 1952.
[9] J. W. Newton, P. W. Wilson, and R. H. Burris, *J. Biol. Chem.*, **204**, 445–451, 1953.
[10] R. M. Allison and R. H. Burris, *J. Biol. Chem.*, **224**, 351–364, 1957.
[11] D. P. Burma and R. H. Burris, *J. Biol. Chem.*, **225**, 287–295, 1957.

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*CELLULAR PROLIFERATION IN THE MOUSE AS REVEALED BY AUTORADIOGRAPHY WITH TRITIATED THYMIDINE**

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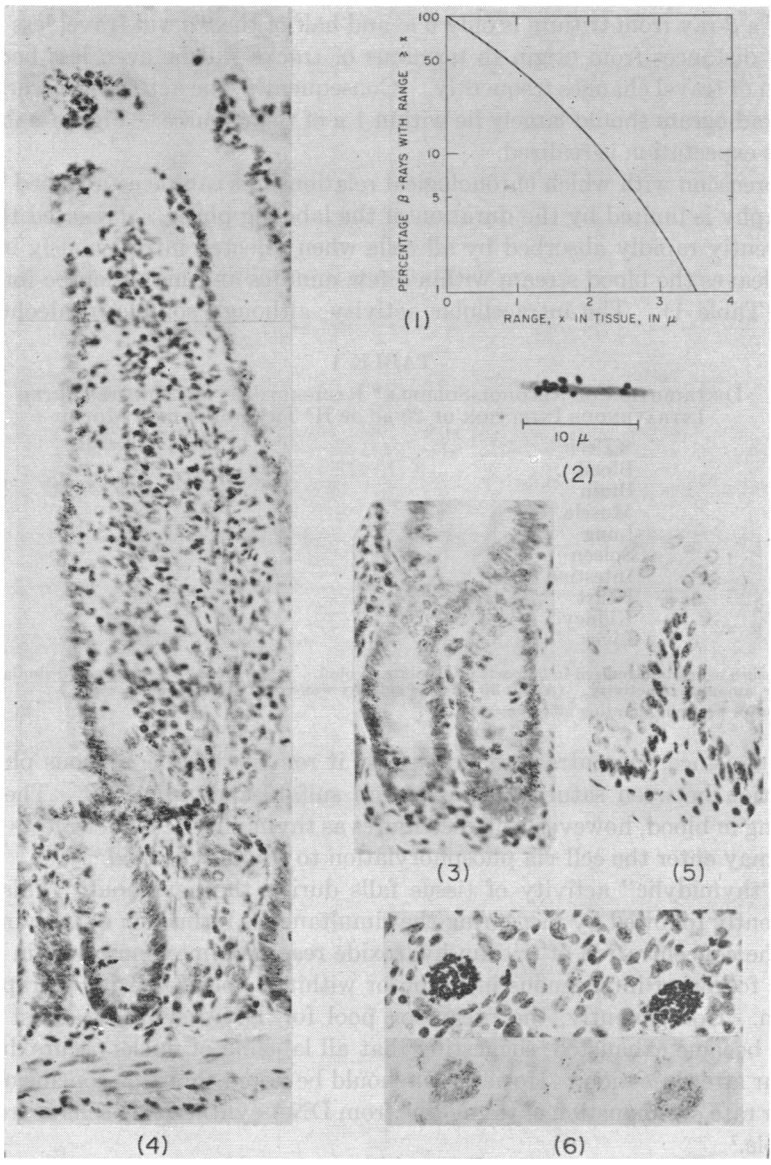
INTRODUCTION

Many cell populations within the body undergo constant breakdown and renewal.[1] Classical histological techniques do not allow adequate study of dynamic processes, and autoradiography is proving increasingly valuable for following these processes at the cellular level. In order to distinguish cell renewal from the renewal of cellular constituents, the label must be incorporated into a fixed component of the cell which is not lost during the cell's lifetime; and present knowledge suggests that a label incorporated into deoxyribonucleic acid (DNA) should be most useful for this purpose.

DNA occurs within the nucleus of the cell associated with the chromosomes and appears, in fact, to be the bearer of the genetic information which each cell passes on to its descendants. Since this information must be handed down through countless generations, extreme immutability of the DNA molecules would seem desirable, and, in fact, evidence is accumulating that DNA in a cell is never replaced. This evidence, as reviewed[2] by Thorell and Smellie, shows that DNA is metabolically inert in resting cells. The possibility of turnover during cellular division, which these authors considered, would appear to be denied by more recent reports. Thus Taylor, Woods, and Hughes[3] used tritiated thymidine (thymine occurs uniquely in DNA, and thymidine is incorporated efficiently and exclusively into DNA[4]) to label the new DNA of bean roots. Autoradiography showed that chromosomes are transmitted to daughter cells as *intact half-chromosomal units*. This suggests that duplication of the genetic material involves the separation of each chromosome into complementary halves and the formation of new complements upon the existing halves as templates. *Escherichia coli* labeled with tritiated thymidine distributed their DNA among their progeny in a like fashion.[5] In both these cases no loss of label from the total population was observed over several cell generations.

Autoradiographic identification of cells should permit a more detailed analysis of cellular dynamics. The specific advantage[6] of tritium for autoradiography lies in the very high resolution which can be obtained because of the very weak energy and consequently short range of its β radiation (Fig. 1). The maximum range in



FIGS. 1–6.—Fig. 1, range-frequency curve for β-rays of tritium (based on data of Robertson and Hughes). Fig. 2, chain of *E. coli* (thymine-requiring mutant) made up of 2 labeled and 1 or 2 unlabeled cells. Note resolution of approximately 1 μ. Fig. 3, jejunum of mouse 7 hours after injection of thymidine (Harris hematoxylin). 175×. Label in crypts only. Fig. 4, jejunum of mouse 48 hours after injection of thymidine (Harris hematoxylin). Label diluted in crypts and distributed along entire villus. Fig. 5, forestomach of mouse 7 hours after injection of thymidine. (Feulgen) Label in basal-cell layer. Fig. 6, polymorphonuclear leukocytes in intestine, 63 hours after injection of thymidine, 48 hours after irradiation (2,000 rad, whole body). (Feulgen) Photographs with and without filter. *Upper photograph*, tissue; *lower photograph*, grains.

tissue of a β-ray from tritium is only 6 μ and half of the β's will travel less than 1 μ. The net distances from origin to terminus of tracks will be even less because the direction of travel changes frequently. Consequently, the activated silver grains of an autoradiogram should largely lie within 1 μ of their source. Figure 2 shows how well this expectation is realized.

The precision with which chronological relationships can be established via autoradiography is limited by the duration of the labeling phase. Tritiated thymidine is apparently rapidly absorbed by all cells when injected intravenously into mice, since it leaves the blood stream within a few minutes and may then be found in all tissues (Table 1). The intracellular activity, although soluble in alcohol, is not

TABLE 1

DISTRIBUTION OF ALCOHOL-SOLUBLE* RADIOACTIVITY 15 MINUTES AFTER INTRAVENOUS INJECTION OF 20 μC OF H^3 THYMIDINE INTO MOUSE

Tissue	Counts† per Mg
Blood	70
Brain	65
Muscle	60
Lung	60
Spleen	70
Intestine	75
Heart	80
Kidney	130
Liver	260

* The tissues were homogenized in 50 parts of absolute alcohol. Ten per cent TCA extracted similar, but somewhat larger, amounts of activity. (About 30 c/mg of activity was volatile, presumably water.)

† At about 5 per cent counting efficiency.

thymidine, since, in contrast to thymidine, it remains in the aqueous phase upon distribution between saturated ammonium sulfate and *n*-butanol. The activity remaining in blood, however, still distributes as thymidine. This suggests that thymidine may enter the cell via phosphorylation to thymidylic acid.

The "thymidylic" activity of tissue falls during the next hour. This may be conveniently followed by measuring the simultaneous formation of tritium hydroxide. The concentration of tritium hydroxide reaches a maximum within about 45 minutes following intravenous injection or within 1 hour following intraperitoneal injection. Consequently, the precursor pool for DNA synthesis must simultaneously become exhausted, suggesting that all labeling of nuclei occurs during the first hour after injection. However, it should be noted that this conclusion implies a similar rate of exhaustion of thymidine from DNA-synthesizing cells as from tissue as a whole.[7]

The following observations are based on autoradiographic examination of twelve mice, no two of which received identical treatment. The results, though obviously preliminary, seem sufficiently exciting to warrant this communication.

MATERIALS AND METHODS

Tritiated thymidine of a specific activity of approximately 300 curies/mole was obtained from the Schwarz Laboratories. It was labeled in the pyrimidine portion of the molecule (presumably by exchange with that hydrogen bound to carbon in the pyrimidine ring), and it was free of labeled impurities as judged by recrystallization with carrier thymidine. It was diluted with isotonic sodium chloride to a convenient concentration for injection.

Young adult male C57 brown mice were injected intraperitoneally with 0.7 μc of tritiated thymidine per gram mouse. Animals were sacrificed at intervals, having been fasted for 12 hours previously. (This brief fasting period yields intestines which are nearly empty and does not seem to affect cell production in the gastrointestinal tract; we are not certain about other organs.) Tissues were fixed in neutral formalin, imbedded in paraffin, and sectioned. Autoradiograms were made with stripping film as described by Pelc[8] and developed after 1–2 months' exposure. Staining was with Feulgen before autoradiography or with Harris hematoxylin after photographic development. Autoradiograms so prepared show essentially no grains except over labeled nuclei.

Six normal mice were sacrificed from 7 to 48 hours after injection. Three mice were X-irradiated 7 hours after labeling and three, 18 hours afterwards; these mice were sacrificed 7–47 hours after irradiation. X-irradiations were administered to the whole body with 250-kvp X-rays filtered through $^1/_4$ mm. of Cu + 1 mm. of Al, at a target distance of 50 cm., with a mean dose rate of 156 rads/min. An average dose of 2,000 rads was administered. This dose leads to acute intestinal radiation death in all mice.

OBSERVATIONS ON NORMAL ANIMALS

Gastrointestinal epithelia.—The epithelial lining of the various portions of the gastrointestinal tract is continually renewed. The zones where cells originate, spend their functional life, and finally end by being desquamated into the lumen occupy separate regions, which are, by and large, well known.

Seven hours after intraperitoneal injection of the thymidine solution, labeled cells were found in the generative zones: the basal layer of the forestomach (which is lined with stratified epithelium) (Fig. 5), the gland necks of the fundus, the crypts of the pyloric region (particularly the lower third, except for the cells at the very bottom of the crypts), the crypts of the small bowel (including the region occupied by the Paneth cells; however, no label was seen in cells containing granules) (Fig. 3), the crypts of the colon. The fraction of labeled cells ranged from about one-fifth in the forestomach, through one-third to one-half in stomach and colon, to about two-thirds in the jejunum. The amount of label per cell appeared to be fairly uniform. Most mitoses seen at this time were strongly labeled (in a subsequent series, labeled telophases were found as early as $2^1/_2$ hours after injection).

In preparations taken 14–48 hours after injection, labeled cells were seen to have moved from the generative to the functional zones; concomitantly, the number of labeled cells in the generative regions decreased, as did the amount of label per cell (Fig. 4). An exception seems to be the colon, where a moderate number of heavily labeled cells remained near the bottom of the crypts after 48 hours. The movement of labeled cells was not very regular. This was best seen in the jejunum, with its large percentage of labeled cells: the zone behind the leading edge was characterized by patchy labeling, and the distance to which the label progressed varied considerably among villi and even on the two sides of a single villus. A substantial fraction of the cells appears to pass through a "pool" which is composed of the intestinal lining between the mouths of the crypts and the bases of the villi. Cells passing through this pool migrated up the villus in a wave, which seems to be about 15 hours behind the leading edge.

In fundus, pylorus, jejunum, and colon the distance from generative zone to surface was covered in less than 2 days, as shown by the appearance in the lumen of desquamated labeled cells 48 hours after injection (Fig. 4). In the same 48 hours, in the forestomach, the label moved into the prickle-cell layer but did not reach the surface. In the depth of the fundus glands, a few labeled cells were found at this time but no fully differentiated chief and parietal cells. In the forestomach and fundus, the life-span of the adult cells is seen to be more than 2 days.

The percentage of labeled mitoses was nearly 100 in the preparation taken 7 hours after injection; it was very low 14 hours after injection and showed irregular variations (between 20 and 60 per cent) in subsequent samples.

Spleen.—The most heavily and frequently labeled cell was the normoblast; the label seemed to stay constant for some time but appeared reduced at 48 hours. Labeled megakaryocytes were found in all preparations, the percentage labeled possibly increasing with time up to about 60 per cent. In the Malpighian follicles, a few reticulum cells and about 10 per cent of the lymphocytes were labeled; there were some groups of labeled cells in the germinal centers. The amount of label in lymphocytes was smaller than in the generative cells of the gastrointestinal tract. During the first day after injection, the percentage of labeled lymphocytes increased and had decreased at 48 hours. A few doughnut-shaped myelocytes were labeled in all specimens; at 48 hours, there were large numbers of labeled mature granulocytes.

Other Tissues.—In the first series of animals, the pancreas contained label only in some duct cells; in a later series (not fasted) several well-labeled cells and mitoses were found. Occasionally, fibroblasts, and, rarely, smooth-muscle cells were labeled in all preparations. A labeled liver cell was infrequently found. An occasional labeled cell was found in Brunner's glands.

OBSERVATIONS ON X-IRRADIATED ANIMALS

In the intestine, labeled cells contributing to the intestinal debris were seen in all irradiated animals. Nuclear debris in lymph follicles and germinal centers was unlabeled in animals X-irradiated 7 hours after injection and were labeled in one animal X-irradiated 18 hours after injection. In one animal sacrificed 2 days after X-irradiation, there was a striking number of labeled macrophages (Fig. 6).

In the gastrointestinal tract the migration of the labeled cells from the generative zone to the surface was not markedly affected by irradiation with 2,000 rads. Also, the maturation and function of the leukocytes did not seem to be affected, since many labeled leukocytes were found inside the glands of the stomach and intestines at 48 hours (Fig. 6).

Discussion.—These results indicate that tritiated thymidine is a powerful tool for studying physiological processes involving the proliferation, maturation, and migration of cells in normal and pathological states.

Nuclei labeled with tritiated thymidine appear to be marked permanently, subject only to dilution of the label by cell division. Factors that might cause underestimation of the number of labeled cells are (1) dilution of the label by so many cell divisions that the label would escape detection (under the conditions of the present experiments this might occur after three or four divisions); (2) loss of tritium by exchange with water. Such loss appears improbable because the tritium in the labeled thymidine is stable in water even in the presence of acid or alkali.

The quantitative estimation of radioactivity in each nucleus by grain counts would be useful in determining the number of cell divisions subsequent to labeling, since each division should halve the radioactivity per nucleus. However, the technical difficulties are considerable. The very short range of β-rays from tritium limits the average number of silver grains activated per disintegration to approximately 1 with tritium dissolved in the emulsion. In actual practice, geometrical factors sharply lower autoradiographic efficiency; we estimate that there is about 1 grain per 5 disintegrations for labeled bacteria and about 1 grain per 20 disintegrations for labeled nuclei in smears of mammalian cells. The low yields of grains can be compensated, to a degree, by long exposure times because the half-life of tritium is long (about 12 years). In tissue sections the situation is further complicated by the fact that the thickness of the section is several times the range of most of the β-rays. Therefore, labeled loci not very near the cut surface may not register at all. The number of grains showing above a labeled cell will depend on both the amount of label in the cell and the distance between it and the emulsion; this distance, in turn, depends not only on interposed tissue but can also be significantly altered by unavoidable imperfections in apposition of stripping film to tissue. Accordingly, estimates of the amount of label in cells in section can be made only in comparison with other cells situated in the same plane and are never precise. More quantitative studies by grain count may be possible by the study of imprints and smears where the geometry is more favorable.

One has also to consider the possibility that labeled cells may not behave like normal ones. In the experiments reported here, the grain count over maximally labeled cells (about 20 grains after 2 months' exposure) indicated that they contain of the order of 10^5 atoms of tritium per cell. This calculation is based on the fact that tritium disintegrates at the rate of 0.5 per cent per month and assumes that there are about 50 disintegrations per grain formed. The labeled thymine was therefore a small fraction of the total thymines (about 10^{10}) contained in a nucleus.

We can further estimate that the amount of thymidine (labeled and unlabeled) added in our experiments must have been diluted one thousand fold in the cell during its conversion into new DNA. Hence it appears that the added thymidine could not significantly affect the size of the thymidine pool or the kinetics of thymidine incorporation into DNA.

On the other hand, one cannot exclude the possibility that radiation from tritium has altered cellular physiology. A cell labeled as in our experiment experiences about 1 disintegration per hour, and the average 5.7 kev of energy liberated (the equivalent of 200 ion pairs) is largely absorbed within the nucleus. We have not noticed any marked effect of the label upon subsequent cell divisions or cell survival; but our study has been limited to periods up to 2 days and to a few kinds of cells. In tissue culture of HeLa cells, labeling levels at least one hundred times higher appear necessary to inhibit cell proliferation.[9]

In cell populations which are homogeneous with respect to cell type and are synchronized, the evaluation of labeling studies is straightforward. In mammalian tissues these conditions are not fulfilled but can be approximated by producing certain conditions, such as regeneration, or by considering only cells at a particular well-defined stage, such as mitosis. All cells labeled by a single brief exposure to tritiated thymidine must have been synchronized in the sense that they were forming DNA at the time of labeling.

If a cell population is homogeneous and completely asynchronous, then the percentage of labeled cells present after a brief labeling period measures the percentage of the total generation time during which cells can take up thymidine and use it for DNA synthesis. If the generating zones of the gut are thus considered as homogeneous populations of cells, it follows from our results that the synthetic time must vary from 20 to 70 per cent of the generation time in different regions. (In tissue cultures of bone marrow[10] or HeLa cells[11] the synthetic time is approximately 30 per cent of the generation time.) If the durations of the periods of synthesis in these different regions are similar, then the rate of cell renewal must vary directly with the fraction of labeled cells. In agreement with this, labeled cells moved more rapidly out of the heavily labeled crypts of the jejunum than out of the more sparsely labeled basal epithelium of the forestomach.

With regard to intensity of labeling, throughout the gastrointestinal tract those cells that were labeled at all appeared to be labeled with equal intensity, also suggesting similar synthetic rates. By way of contrast, lymphocytes were weakly labeled. This may be interpreted as possibly indicating a slower synthetic rate for the lymphocyte. However, in addition to the inaccuracies resulting from variation in autoradiographic efficiency, variation in cellular uptake of the isotope or in size of the precursor pool might explain this difference.

The time required for the appearance of labeled mitotic figures following injection measures the interval between completion of DNA synthesis and the beginning of mitosis. Since all mitoses were labeled 7 hours after injection (the shortest time studied), all cells must proceed into mitosis within 7 hours following the completion of DNA synthesis. In the subsequent intervals studied, the percentage of labeled mitoses decreased to a low value at 14 hours and then varied between 20 and 60 per cent. It is possible that further studies over more frequent time intervals may show a rhythmic variation in labeling, which should correspond to the generation time.[10]

Radiation injury affects the gastrointestinal tract by stopping the renewal of its epithelia. With a sufficiently high dose, the generative compartments are largely emptied, and the production of new cells is blocked. Death may ensue if the functional compartment is depleted before regeneration can provide replacement. It has been postulated that the generative compartment is largely emptied by *cell death* and that the functional compartment remains populated, for a time, with those cells which were in it at the time of irradiation.[12] This picture must be modified on the basis of the present studies: the generative compartment is emptied not only by cell death but also by cell migration, since cells continue to migrate into the functional compartment after 2,000 rads of X-irradiation.

Summary.—Tritium carried in thymidine has been used to label cell nuclei of mouse tissues by incorporation into deoxyribonucleic acid. Autoradiograms show surprisingly large numbers of labeled nuclei, suggesting that the amount of cellular proliferation is greater than has been inferred from the study of mitotic indices. The period for deoxyribonucleic acid synthesis appears to be a large fraction of the generation period in the intestinal mucosa. Cellular migration in the gastrointestinal epithelium from the crypts to the tips of the villi is clearly demonstrated by this technique, and variations in the rate of migration are seen in different regions. Two thousand rads of X-rays selectively inhibit cell production without markedly

affecting the maturation or migration of cells into functional compartments. The possibilities and limitations of tritiated thymidine for further studies are discussed.

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[1] C. P. LeBlond and B. E. Walker, *Physiol. Rev.*, **36**, 255, 1956.

[2] E. Chargaff and J. N. Davidson, *The Nucleic Acids* (New York: Academic Press, 1955), Vol. II, Chaps. xx and xxv.

[3] J. H. Taylor, P. S. Woods, and W. L. Hughes, *Proc. Natl. Acad. Sci.*, **43**, 122, 1957.

[4] P. Reichard and B. Estborn, *J. Biol. Chem.*, **188**, 839, 1951; M. Freidkin, D. Tilson, and D. Roberts, *J. Biol. Chem.*, **220**, 627, 1956.

[5] R. B. Painter, F. Forro, and W. L. Hughes, *Nature*, **181**, 328, 1958.

[6] P. J. Fitzgerald, M. L. Eidinoff, J. E. Knoll, and E. P. Simmel, *Science*, **114**, 494, 1951; J. S. Robertson and W. L. Hughes, *Trans. First Natl. Conf. Biophys.*, 1958.

[7] L. D. Hamilton has suggested that phagocytes might re-utilize the DNA from a single cell; this would result in delayed labeling of the phagocyte (see "Homeostatic Mechanisms," *Brookhaven Symposia Biol.*, 1957).

[8] S. R. Pelc, *Nature*, **160**, 749, 1947.

[9] R. B. Painter, R. M. Drew, and W. L. Hughes, *Science* (in press).

[10] L. G. Lajtha, R. Oliver, and F. Ellis, in *Radiology Symposium 1954*, (London; Butterworths), and *Brit. J. Cancer*, **8**, 367, 1954. See also L. G. Lajtha, R. Oliver, T. Kumatori, and F. Ellis, *Radiation Research*, **8**, 1, 1958.

[11] R. B. Painter, unpublished observations.

[12] H. Quastler, *Radiation Research*, **4**, 303, 1956; G. Brecher, E. P. Cronkite, R. A. Conard, and W. W. Smith, *Am. J. Pathol.* **34**, No. 1, 105–119, 1958.

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*DIFFERENCE IN ELECTRIC POTENTIAL ACROSS THE PLACENTA OF GOATS**

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The data presented in this report were obtained during part of an investigation[1] directed primarily to study the "forces" that determine the net transfer of water across the placenta from the maternal to the fetal blood and the mechanisms that regulate the water content of the fetus and the surrounding cavities. To learn whether electro-osmotic phenomena operate in the process of transfer of water across the placenta, we have proceeded to determine whether or not any difference in electric potential exists across the barrier that separates the maternal from the fetal blood.

The animals used for the experiments were goats (duration of pregnancy 145–47 days), bred on known dates. The heparinized mother was under spinal anesthesia (Pontocain) and received, in addition, a light dose of thiopental sodium (Pentothal)