PROLIFERATIVE ACTIVITY OF THE LYMPHATIC TISSUES OF RATS AS STUDIED WITH TRITIUM-LABELED THYMIDINE*

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Isotopic labeling of the DNA of blood cells has provided an important technique for obtaining information about the cytokinetics of white cells. It has been assumed that the DNA label is stable within a given lineage of cells. However, several papers have indicated reincorporation of tritiated thymidine (H^3Tdr)–labeled deoxyribonucleic acid (H^3DNA) of cells during catabolism by other proliferating cells. The tritiated thymidine is originally incorporated during DNA synthesis (1–6). Most interpretations of this finding have assumed that it is not the result of radiobiological artifact due to the isotope. Aside from the significance of this finding in terms of interpretation of data pertaining to cell renewal, life span, and possible transformation of blood cells into different morphological entities (7), the possibility of the DNA within cells being unstable has many other implications.

The biochemistry of the transfer of DNA label remains to be elucidated. Hamilton (8), whose work in leukemic subjects led him to propose that DNA reutilization might occur within lymphatic tissue, believed that his data favored the reincorporation of fragments of DNA polymer rather than mononucleotides or nucleosides resulting from DNA breakdown. He pointed out that the earlier works of Ottesen (9) and Osgood *et al.* (10) were also consistent with this view. On the other hand, Robinson and Brecher have recently reported strong evidence suggesting that reincorporation of the DNA label in blood cells by rapidly growing liver cells occurs after breakdown of DNA to a nucleoside level (11).

After flash labeling of the DNA of a portion of a normally replicating cell population which is in DNA synthesis at the time the H^3Tdr is available, dilu-

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tion of the DNA label in the divisible cells, as new DNA is synthesized during repeated cell divisions, should follow a single first-order decline. This is not the case in some tissues (12–14). The reasons for this are not clear but could reflect some damaging effect of the isotope on a portion of the DNA which has incorporated the H^3Tdr, prolonged interphase of some cells in a given population, prolonged retention of label in an early, "stem" cell population feeding cells into the particular proliferating pool under study, or inherent asynchrony of DNA replication in some cells. Another more likely possibility, in view of the evidence that the DNA label can be transferred from blood cells to such tissues as replicating liver (1, 5), skin (2), and tumor cells (4) is that the persistence of some DNA label reflects reutilization of the DNA label by growing cells. This interpretation has been given by others to events in lymphatic tissue (14) and more recently for the intestinal mucosa (3). It has been assumed that the source of DNA label to be reincorporated in such tissues as the intestinal mucosa is from desquamating mucosal cells containing the DNA label. Another possible source of reincorporated label is that contained in lymphatic tissue and in white blood cells moving through the area.

The purpose of this paper is to present findings on the course of H^3Tdr labeling of DNA in various parts of the lymphatic tissue of young adult male rats. It has been assumed by the authors that if the label in DNA after H^3Tdr administration can be transferred from blood cells to other proliferating tissues, the same process can occur between lymphocytes in lymphatic tissue. The persistence of DNA label in a precursor population of cells is accepted as evidence for this occurrence. It should be emphasized that the term DNA label, as employed here, refers to material in the nucleus of replicating cells which becomes labeled with H^3Tdr, is extractable from tissues by procedures commonly employed to remove DNA, contains thymidylic acid, and lacks uridylic acid. There is some evidence that DNA is heterogeneous (15) and there is no way of knowing, in the present work, whether H^3Tdr labels only that DNA in the chromosomes which is genetically determinative.

Materials and Methods

These studies have employed simultaneous radiochemical and autoradiographic determinations of DNA labeling of various tissues of rats after administration of H^3Tdr. Autoradiography has definite limitations of interpretation, particularly in fixed tissue sections, as pointed out by Cottier (16). Since much of the present data deals with low levels of DNA labeling per cell, radiochemical assessment of tissue DNA label has been of value in establishing the degree of labeling in certain tissues. The deficiencies of radiochemical techniques in the analysis of DNA labeling in tissues of mixed cell species are well known, but coupled with autoradiographic localization of the labeled cell population, they have been of assistance.

Radiochemical Techniques.—The tissues were weighed and aliquots dried in plastic bags, reweighed, and combusted. The total dry tissue H^3 content was determined as tritiated water of combustion (17). This provided a measure of any H^3 label in DNA and in non-DNA or acid-soluble nucleoside or nucleotide pools. This technique was preferred to analysis of RNA

and acid-soluble nucleotides because of its simplicity, the high percentage recovery of tissue H^3 and the avoidance of variation in nucleic acid and nucleotide extraction of different tissues.

The DNA of a portion of tissue was extracted and quantitated as described previously (18). Samples containing any non-DNA phosphorus were discarded. Aliquots of DNA extracted from each tissue were hydrolyzed and the bases chromatographed. Any DNA containing detectable uracil contamination after hydrolysis and chromatographic separation of the bases was discarded.

All radioactivity determinations were performed in the same instrument (tri-carb, Packard Instrument Co., La Grange, Illinois) under identical conditions. Determinations of quench were made for DNA from each tissue and for DNA of varying concentrations. The tritiated water samples, obtained from combustion of dehydrated tissue, were counted in a suspension-scintillator system consisting of PPO, POPOP,[1] toluene, and ethyl alcohol. The DNA extracted from tissues was dissolved in water and counted in a gel-scintillator consisting of PPO, POPOP, thixcin, and hyamine chloride in toluene. The volumes of water counted were either 0.1 or 0.2 ml in 20 ml of solvent or gel-scintillator.

Samples of tissue to be combusted were divided into three aliquots and combusted separately. Tissues analyzed include bone marrow, small and large intestine, adrenal, liver, kidney, and lymphatic organs. Results will be given for lymph nodes, thymus, and spleen. Lymph nodes included were all those visible in the mediastinal, axillary, periaortic, and mesenteric regions and tissue from each area was combusted separately. These nodes were assumed to be representative of the peripheral organized lymph nodes. No attempt was made to quantitate the entire body lymphoid tissue. Samples containing any color, a difficulty encountered only with bone marrow and whole blood, were discarded. Samples which were incompletely combusted with any remaining ash were discarded. Each of three samples from the same tissue, except in the case of lymph nodes, was counted for three 10 minute periods and checked within 10 per cent. Repeat counts of the same sample varied within 1 per cent. For the lymph nodes, individual nodes from each area selected were weighed and combusted separately. The largest methodological variation with this technique concerns the hydroscopic action of the plastic bags containing the dehydrated samples. All weighing was performed on a Mettler electrical balance (model M-5) accurate to $\pm$ 0.002 mg. Variation in bag weight after drying was controlled in each instance by weighing an empty dried bag and subtracting the final bag weight from that of the sample.

Quadruplicate H^3DNA samples were counted from each tissue, including the pooled lymph nodes. Variation was within 5 per cent and repeat counts of the same sample varied within 1 per cent. Although the variation from animal to animal within a group sacrificed at the same time after the same dose of H^3Tdr was considerable, sufficient points in time were obtained to establish clear-cut trends for initial uptake into DNA and loss of H from DNA with time for each organ studied. The differences between tissues with a rapid DNA turnover such as erythroid and myeloid bone marrow and intestinal mucosa and slowly replenished structures such as liver, kidney, lymph nodes, etc., are very marked. Data concerning tissues other than the lymphatic system will be presented in a subsequent paper.

Autoradiographic Technique.—Touch preparations of freshly cut cross-sections of spleen, lymph nodes, and thymus were made on gelatin-coated slides. Marrow smears and imprints for autoradiography were fixed in Carnoy's fixative which removes any H^3 in acid-soluble pools from the slide. Duplicate portions of tissues to be sectioned at 4 micra were fixed in Carnoy's solution and formalin for 4 hours. Autoradiography was performed with the stripping film technique as described previously (18). Staining was with acid-Giemsa as described by Feinen-

[1] PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene. Purchased from Packard Instrument Co., La Grange, Illinois.

degen and Bond (19). Duplicate sets of slides were developed after 2 and 4 weeks. Sections of each tissue were also stained with hematoxylin and eosin and with methyl green pyronine.

Tissue and cell morphology and localization of the DNA label were studied in the following tissues: marrow, blood leukocytes, small intestine, large intestine, liver, kidney, adrenal, axillary, mediastinal, mesenteric and periaortic lymph nodes, thymus, and spleen. Quantitation of grains will be described only for the lymphatic organs and only for lymphoid cells in these organs. Quantitation of grains is more reliable on imprint material with the technique employed and the data presented for sequential dilution of DNA label in large lymphocytes is from imprints of thymus and lymph nodes. Imprints of spleen have the disadvantage of including variable numbers of immature myeloid and erythroid cells which are at times difficult to distinguish from immature lymphoid cells in spleen red pulp and bone marrow preparations. For this reason the data on mean grain counts of large lymphocytes and thymocytes are derived from imprints of lymph nodes and thymus.

The lymphoid cells of the lymph nodes and thymus have been divided into small, medium, and large, following the criteria of others (20). However, there are several gradations of medium-sized cells and, for the purposes of the data pertaining to the rate of grain dilution as an indication of the rate of DNA renewal, only the largest lymphocytes were scored. The large cells of the thymus cortex and in the medullary cords and cortices of lymphoid follicles stand out because of their size and their staining characteristics. The nucleus is considerably paler and the cytoplasm much darker than in the surrounding small cells. The cytoplasm of these immature cells is uniformly more pyrinophilic than is that of smaller, presumably more mature lymphocytes.

Background scatter on imprint material was determined by counting the grains per 1000 micra^2 over portions of the slide adjacent to cells, taking the mean of 10 such areas. This value was subtracted from the mean grain count so that the values given are relative to background. The range of background grains was from 0 to 3 per 1000 micra.^{2} One hundred large thymocytes or lymphocytes were counted on each of four imprints of the thymus and lymph nodes. One thousand cells were counted to determine the per cent labeled cells.

In the case of the spleen, where only section material could be employed because of the mixed cell population, the radioactivity in different regions of the tissue could be readily estimated over groups of cells, but accurate grain counts of lightly labeled cells were not possible. The spleen material was employed, therefore, to provide localization of highly labeled (10 grains or more above background), lowly labeled, or unlabeled lymphoid cells. The comparative degree of DNA labeling of lymphoid cells in the germinal centers, cortex of lymphoid follicles, and those in the splenic red pulp was estimated using these rough criteria. Germinal centers are defined, for the purposes of this paper, as the collections of lymphoid cells near the center of the spherical follicle and adjacent to the small blood vessel with characteristically high endothelial lining cells (21). These cells have a higher mitotic index than cells elsewhere in the follicle (22–25). These cells are mostly large- and medium-sized cells.

Animals.—Sprague-Dawley male rats bred in the rat colony of the Department of Biological Chemistry, UCLA Center for Health Sciences, Los Angeles, were employed. These rats increase their weight at a rate of 2 per cent per day between 100 and 350 gm. The increase thereafter is more gradual and consists mainly of additional body fat. Heavier animals, receiving the same dose per gram of H^3Tdr as smaller animals, show an increased uptake of the isotope into the DNA of proliferating tissue because of the dose is influenced by the amount of slowly replicating adipose tissue. It is important, therefore, to select animals less than 350 gm body weight for this type of study. All animals were between 150 and 250 gm unless otherwise specified.

Animals were sacrificed serially by exsanguination. Partial hepatectomy (40 to 50 per cent) was performed by the technique of Higgins and Anderson (26).

Isotopes.—H^3Tdr, 6.4 c/mmole was obtained from New England Nuclear Corp., Boston. Injections were given in volumes of 0.25 to 1.0 ml subcutaneously to unanesthetized animals at the same time of day, usually between 7 and 8 a.m. Intravenous administration of the isotope to 10 rats and 24 mice provided an initial uptake of H^3Tdr into the DNA of various organs which was not significantly different from animals receiving subcutaneous injections. The amount of isotope administered to the various groups of animals is indicated in the results.

The C^{14} formate employed had a specific activity of 9.5 mc/mmole.

RESULTS

The initial uptake of H^3Tdr into thymus DNA is approximately one-fifth that of the peripheral lymph nodes and one-eighth that of the spleen. These findings, similar to those of Nygaard and Potter with C^{14}Tdr (13), are shown in Table I

TABLE I

Comparison of H^3 in DNA, As Determined by DNA Extraction, and H^3 in Whole Tissues of 36 Normal Rats

Average weight 200 gm, 1 to 5 hours after H^3Tdr, 0.5 μc/gm.

Organ	H^3 content of dry tissue	Concentration of DNA-P in fresh tissue*	H^3DNA, from DNA extraction of tissue	H^3DNA calculated from H^3 content and DNA-P concentration‡
	CPM/mg ± 1 SD	mg DNA-P per mg × 10^6	CPM/mg DNA-P × 10^{-3} ± 1 SD	CPM/mg DNA-P × 10^{-3}
Liver	38.2 ± 6.6	21–25	36.2 ± 3.2	38.4–45.7
Kidney	62.4 ± 8.6	33–34	58.1 ± 5.3	45.8–47.2
Spleen	830.2 ± 182.6	76–85	170.0 ± 22.1	195.3–218.4
Lymph nodes	426.1 ± 262.2	76–85	91.4 ± 24.1	100.3–121.3
Thymus				
1-dose	208.2 ± 58.2	181–242	20.1 ± 6.2	17.2–23.0
3-dose§	1070.0 ± 55.0	181–242	115.3 ± 18.5	85.1–118.8

* From Davidson, The Biochemistry of Nucleic Acids (53).

‡ Dry weight 25 per cent of wet weight for liver and kidney. 20 per cent of wet weight for spleen, lymph nodes and thymus.

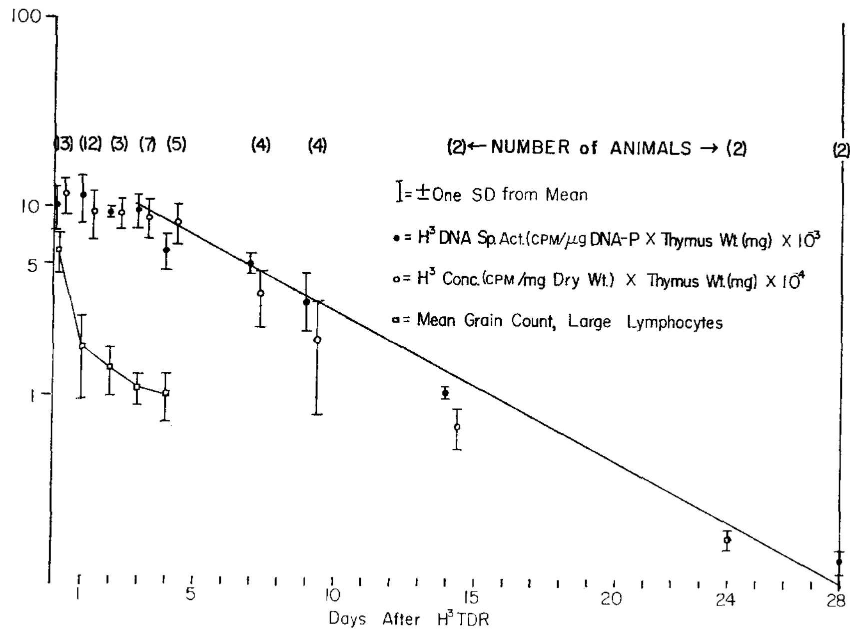
§ Four rats sacrificed 1 to 24 hours after last of 3 doses of 0.5 μc/gm at 24-hour intervals.

and Text-figs. 1 and 2. In spite of variation from animal to animal, there is good agreement between the H^3DNA specific activity and the total H^3 in the dehydrated tissue. Calculating H^3DNA from H^3 content, known DNA-P concentration, and wet weight to dry weight ratio, provides values comparable to the measured H^3DNA label. This was true in animals receiving a single dose of 0.5, 1.0, and 0.5 μc/gm at 24 hour intervals for 3 doses.

Text-fig. 1 shows a plateau of thymus radioactivity for 3 days after administration of a single dose of H^3Tdr. Fifty-six to 72 per cent of the large thymic lymphoid cells were labeled in these normal rats sacrificed 1 to 5 hours after a single dose of H^3Tdr. There is rapid dilution of the H^3DNA per large thymocyte, as shown by the fall in mean grain count during the first 24 hours. Thereafter,

the drop in mean grain count per large cell is more gradual, although after 0.5 μc/gm, the grains per cell after 48 hours are too low to permit accurate quantitation. Serial grain counting of large thymic cells in rats receiving 1 μc/gm, in which the initial mean grain count was twice as high as in these rats receiving 0.5 μc/gm, confirms the more gradual fall in grains beyond 24 hours.

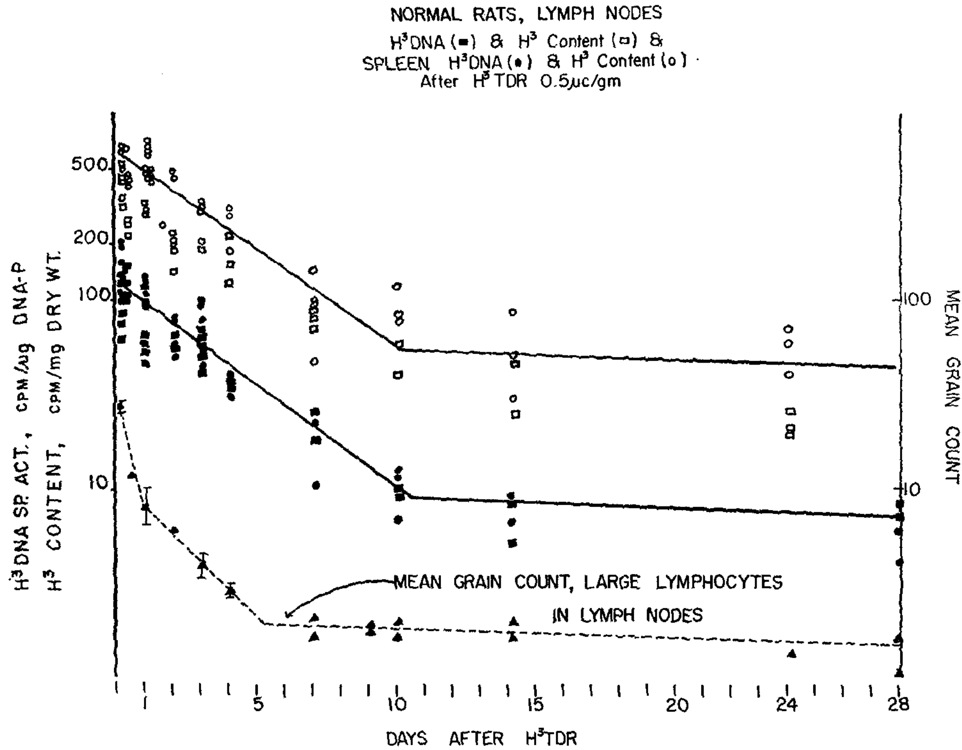
The peripheral lymph node and spleen H^3DNA and H^3 content data are shown in Text-fig. 2. The lines shown are intended to give the best fit to these



TEXT-FIG. 1. The loss of H^3 from normal rat thymus after administration of H^3Tdr, 0.5 c/gm. As shown in Table I, similar values are obtained for H^3DNA specific activity by direct measurement and by estimates based on H^3 concentration. In this and subsequent figures the curves of loss of H^3 from DNA (H^3DNA specific activity) and from the whole tissue (H^3 concentration in CPM/mg dry weight) are the same. There is no detectable H^3 except that in DNA. The mean grain counts refer to the largest thymic progenitor cells which are clearly lymphoid cells, counting 200 cells for each point.

points and the slopes are approximations only. In most instances the label concentration is higher in spleen than in lymph nodes. The decline in radioactivity appears to follow two first-order curves, the first with a half-life of about 3 days. The second slope is very gradual indeed with a half-life of some 50 days. Nygaard and Potter (13), in similar studies of the loss of C^{14}DNA from rat spleen after C^{14} thymidine administration, did not carry their observations beyond 7 days following isotope administration and, therefore, did not observe the very prolonged retention of some DNA label. They noted two separate

decay curves for the spleen during the 1st week, one with a half-life of 1.2 days and a second with a half-life of 4 days. The present studies show so much spread of values from animal to animal that it is not possible to define more closely the pattern during the 1st week after isotope administration. However, if Nygaard and Potter's findings can be combined with these data it is evident that the decay is very complex, consisting of at least three components. The most strik-



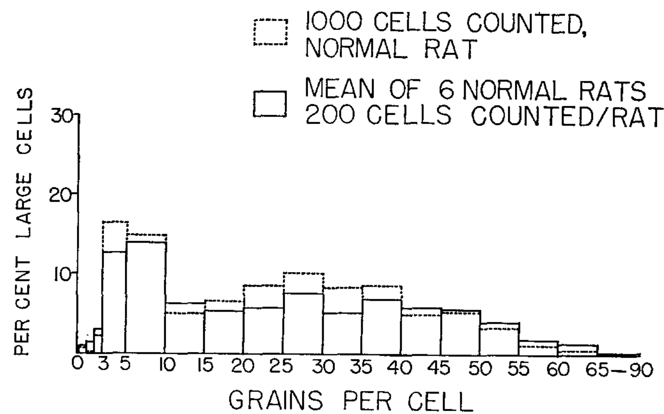
TEXT-FIG. 2. The loss of H^3 from normal rat lymph nodes and spleen after H^3Tdr, 0.5 μc/gm. Also shown is the serial mean grain counts of large lymphoblasts in a mesenteric lymph node.

ing feature of the decay is the very prolonged retention of some DNA label by both lymph nodes and spleen.

The rate of dilution of H^3DNA in large lymphocytes in lymph nodes, expressed as mean grain count, is also shown. Forty-eight to 72 per cent of the large lymphocytes in imprints of normal lymph nodes were labeled in these normal rats sacrificed 1 to 5 hours after a single dose of H^3Tdr. Amongst the labeled large lymphocytes there appeared to be at least two groupings of label intensity with an increased number of cells containing 3 to 10 grains and the *remainder showing the expected distribution about a mean of 35 grains* (Text-

fig. 3). As the large lymphocytes lose label with repetitive divisions the range of grain count becomes progressively narrower as cells with a low label intensity become predominant. There is an initial rapid fall in grain count during the first 24 hours, followed by a more gradual decline, which approximates the loss of H^3DNA from the whole tissue. This curve of loss of H^3DNA from large lymphocytes is similar to previous data of this type obtained by Alpen *et al.* (14). These authors resolved this curve into two components, one of half-time of 20 to 30 hours and another of half-time 40 to 50 days.

Rats sacrificed serially after the last of 3 doses of 0.5 μc/gm given at 24 hour intervals (hereafter called 3-dose animals) show a biphasic loss of radioactivity



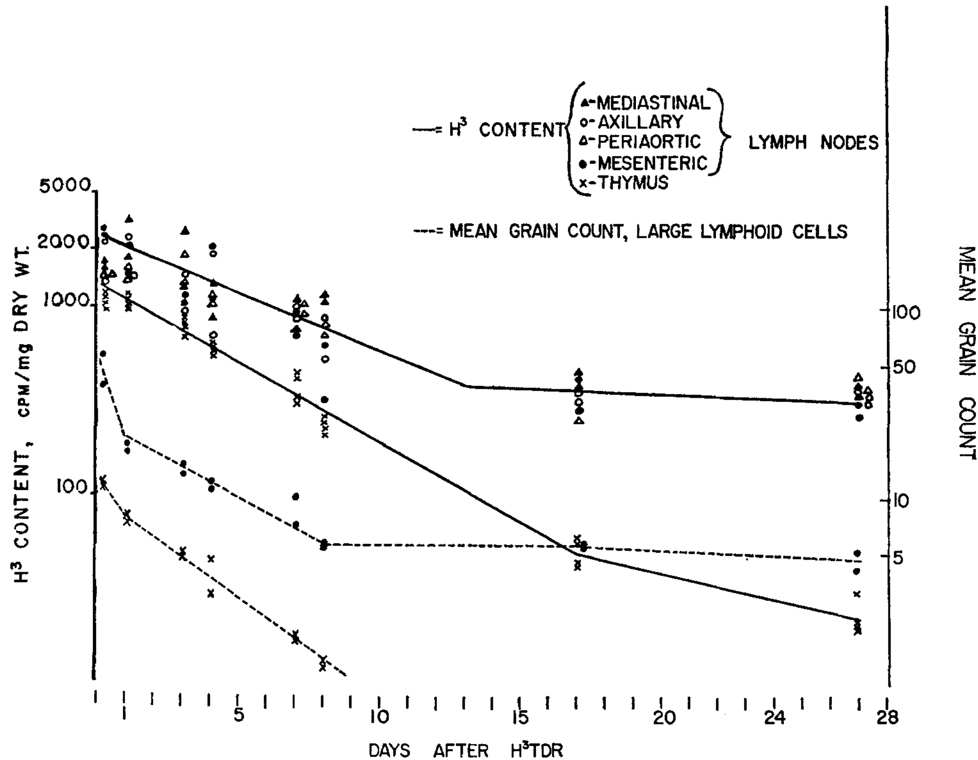
TEXT-FIG. 3. The distribution of grains in large lymphoblasts in imprints of mesenteric lymph nodes of normal rats sacrificed 1 to 5 hours after H^3Tdr, 0.5 μc/gm. All cells in this category are counted.

from spleen and lymph nodes (Text-fig. 4). Sufficient points have not been obtained to determine with accuracy the slope of the more gradual portion of this curve, but the half-time would appear to be of the order of 50 days. Again, the dilution of grains in large lymphocytes is rapid during the first 24 hours after the last dose of H^3Tdr. Thereafter, the fall in grain count appears to parallel the loss of H^3 from the entire tissue.

Text-fig. 4 contrasts the loss of radioactivity expressed as H^3 content from the lymph nodes and thymuses of rats receiving 3 doses at 24 hour intervals. There is more rapid loss of radioactivity from the thymus than from lymph nodes.

Twenty-four hours after a single dose of H^3Tdr, highly labeled small cells, resulting from division of highly labeled large- and medium-sized cells, are found in the follicles of lymph nodes and spleen. The small cells of the thymus are labeled much less intensely. In animals sacrificed serially, highly labeled small lymphocytes such as those in lymph nodes were found in gradually diminishing numbers in all tissues examined except the thymus.

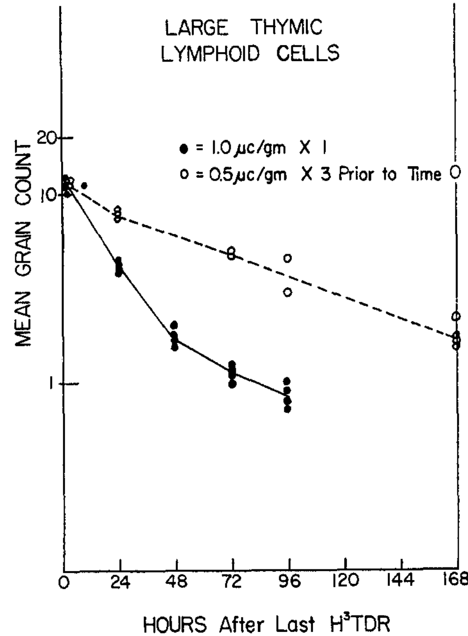
In animals sacrificed 1 hour after the last of 3 doses of H^3Tdr, 0.5 μc/gm at 24 hour intervals, the mean grain count of large progenitor cells in the thymus is about twice as high as it is after a single dose of 0.5 μc/gm. The DNA label was reduced in these progenitor cells during the 24 hours following each injection and the most significant change is the extent to which the radioactivity



TEXT-FIG. 4. Loss of H^3 from normal rat lymph nodes and thymuses after H^3Tdr, 0.5 μc/gm given on each of 3 successive mornings prior to time 0. The first animals were sacrificed 1 hour after the third injection of isotope. The dashed lines describe the mean grain counts of large lymphoid progenitor cells on autoradiographs of thymus and mesenteric lymph node.

has been dispersed to small cells by division of the labeled precursors. In the thymus this appears as low labeling of a majority of the cells in the thymus cortex. The H^3DNA specific activity of the thymus is about 5 times higher in these 3-dose rats than in rats receiving a single dose of 0.5 μc/gm (Table I). It was found that the mean grain count over large thymocytes in 3-dose animals (sacrificed 1 hour after the last dose) was the same as that for animals sacrificed 1 hour after 1.0 μg/gm. The rate of grain dilution in these two groups could be followed starting with the same intensity of DNA label in the large

cells. The results, shown in Text-fig. 5, suggest a more rapid initial fall in mean grain count in animals receiving a single dose, although the number of animals is too small to allow statistical verification. The subsequent rate of dilution of the DNA label is similar in the two groups, though the mean grain count is higher at each point in the 3-dose animals.



TEXT-FIG. 5. Serial mean grain counts of large lymphoid progenitor cells in the thymuses of normal rats after a single dose of H^3Tdr, 1.0 μc/gm (solid circles) and after 3 doses of 0.5 μc/gm given at 24 hour intervals. The first sacrifices were performed 1 hour after the single dose or after the last of 3 doses.

The autoradiographs of section material from these tissues were evaluated for the location of highly labeled, lowly labeled, and unlabeled cells. They consistently showed the presence of highly labeled cells in the medullary cords and peripheral areas of lymphoid follicles of the spleen and lymph nodes. However, the germinal centers of lymphoid follicles of the spleen and lymph nodes, where mitotic activity is highest (22–25), showed cells with a lower label intensity in animals sacrificed 1 to 5 hours after a single dose of H^3Tdr, an example of which is shown in Fig. 1. Similar large lymphocytes in germinal centers of splenic lymphoid follicles with a lower grain count than in more peripherally located large lymphocytes have been described by Fliedner *et al.* (27) and by Cottier *et al.* (28). It is presumed that these cells account for the increased number of cells containing 3 to 10 grains as seen on imprint material (Text-

fig. 3). An unexpected finding in the animals sacrificed at periods of 1 week or more after labeling was the persistence of labeled large and medium lymphocytes in these areas of active cellular proliferation.

Large lymphocytic progenitor cells in the thymus invariably showed a lower mean grain count than large cells in the follicles of lymph nodes and spleen. Figs. 2 *a* and 2 *b* show such cells in imprints from a lymph node and thymus of a normal rat sacrificed 1 hour after a single dose of 0.5 μc/gm. The low uptake

TABLE II

In Vitro Labeling of Thymus and Lymph Node Cell Suspensions from Normal Sprague-Dawley Rats

Rats killed by guillotine. Cell suspensions dispersed in chilled Hanks' solution, particles removed and free cells adjusted to cell concentration of 100,000/mm^3. H^3TDR (6.9 c/mmole) added to each suspension, 20 μc/2 ml, and incubated at 37°C for 2 hours with gentle agitation. Viability determined by eosin uptake.

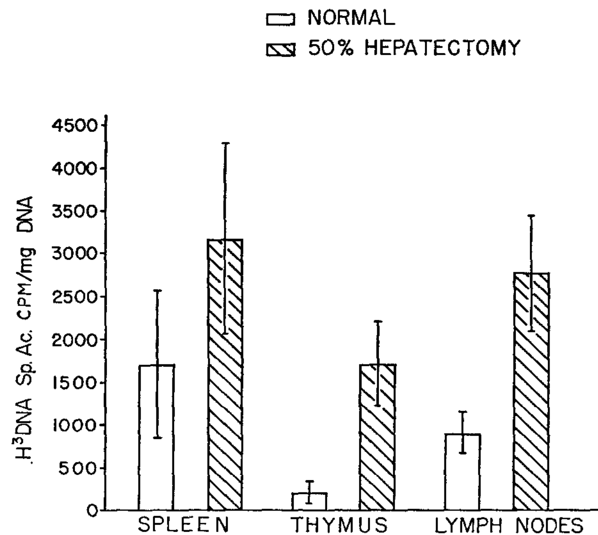
Tissue	Rat No.	Cell suspension			Autoradiographs (1000 large cells counted)			H^3DNA specific activity
		Viable cells	Small	Large	Labeled cells (over 5 grains)		Grains per cell, mean	
					Small	Large		
		per cent	*per cent*	*per cent*	*per cent*	*per cent*		CPM/μg *DNA-P*
Thymus	205	99	92.2	7.9	0.2	53.0	28	261.4
	206	98	89.0	11.0	0.34	62.0	36	
	207	99	93.9	6.1	0.32	58.2	53	
Lymph nodes	205	98	96.7	3.3	0.1	86.7	>100	565.03
	206	98	94.3	5.7	0.21	82.8	>100	
	207	99	96.4	3.6	0.83	94.4	>100	

of H^3Tdr into precursor cells in the thymus cortex, as compared to the majority of precursor cells in lymph follicles (with the exception of those in germinal centers) could be due to differences in blood flow and isotope availability (16). Attempt was made to study this factor employing *in vitro* incubations. Fresh suspensions of dispersed cells were made as quickly as possible after sacrifice and comparable concentrations of cells and isotope incubated under identical conditions. The amount of isotope was purposefully made much higher than would be present *in vivo*. The results, shown in Table II, indicate the lower uptake of H^3Tdr by thymus cells, as compared to lymph node cells.

Rats subjected to partial hepatectomy 3 days before the administration of H^3Tdr, 0.5 μc/gm, and sacrificed 5 hours later, show a marked increase in the uptake of the label into the DNA of lymphoid tissue (Text-fig. 6). The thymus weights in these stressed animals were markedly reduced and the percentage

of small cells in the thymus had fallen from a mean value of 84 to 36 per cent. An example of the intensity of DNA label in the large lymphocytic cells of the thymus is shown in Fig. 3. Similar though less marked changes were noted in the spleen and lymph nodes.

Another group of 10 animals was subjected to lesser and variable degrees of partial hepatectomy. Five days later these rats were given 0.5 μc/gm of H^3Tdr and sacrificed at 2 hours, 1 to 3, 5, 7, and 10 days. It will be noted (Text-fig. 7)

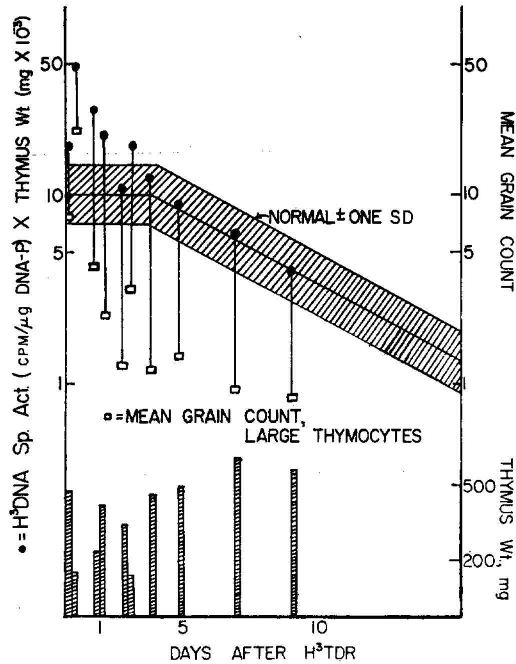


TEXT-FIG. 6. The uptake of H^3Tdr into the DNA of lymphatic tissues of 36 normal rats and 6 rats subjected to 50 per cent hepatectomy 3 days before isotope administration. Animals in both groups received 0.5 μc/gm and were sacrificed 1 to 5 hours later. I, ± 1 SD from mean.

that there was a good correlation between mean grain count of large progenitor cells and total H^3DNA, as estimated by H^3DNA specific activity multiplied by thymus weight. The animals with more severe stress and smaller thymus weight showed a greater uptake of H^3Tdr. In those animals sacrificed within 1 to 5 hours after H^3Tdr there was found to be a good correlation between H^3DNA specific activity, plotted on a logarithmic scale, and the reciprocal of thymus weight (Text-fig. 8 *a*).

As previously described, the mean grain count of thymus progenitor cells falls rapidly during the first 24 hours after a single dose of H^3Tdr. The dilution with subsequent divisions is more gradual (Text-figs. 1 and 5). This same pattern seems to prevail in stressed animals. If the mean grain count of the large thymus progenitor cells in animals sacrificed between 24 hours and 10 days is plotted against total thymus H^3DNA (Text-fig. 8 *b*), a good correlation is found.

These points include animals subjected to variable degress of stress, as noted, and normal animals receiving different amounts of H^3Tdr. It would appear, therefore, that once the DNA label has entered the small cell population of the thymus by division of labeled progenitor cells, the subsequent loss of DNA label from the large cells capable of mitosis occurs at the same rate as the loss of H^3DNA from the whole thymus. Furthermore the late decline in grain count of

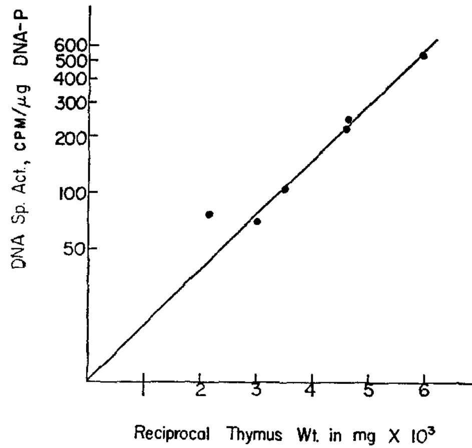


TEXT-FIG. 7. Rats subjected to partial (20 to 30 per cent) hepatectomy 5 days before H^3Tdr administration (0.5 μc/gm) and then sacrificed serially. Although the initial uptake of H^3Tdr is increased, the subsequent loss of H^3 and the dilution of grains in large thymic progenitor lymphocytes is not markedly different from normal.

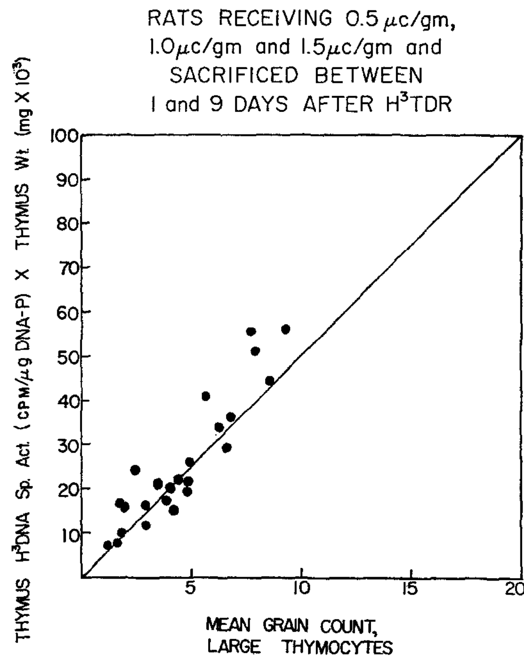
large thymocytes and the late decline in total thymus H^3DNA follow the same approximate slope regardless of the extent of initial labeling of the progenitor cells.

Similar data were obtained on the lymph nodes and spleens of these stressed animals. Over the course of the 9 day period there did not appear to be any significant difference from normal animals for rate of decline of H^3DNA specific activity or mean grain count of large lymphoblasts.

A group of 8 rats, each weighing approximately 140 gm, was given H^3Tdr, 1.0, μc/gm, and C^{14} formate, 0.03 μc/gm, simultaneously. They were sacrificed at intervals and the specific activities of lymph node and thymus DNA C^{14} and

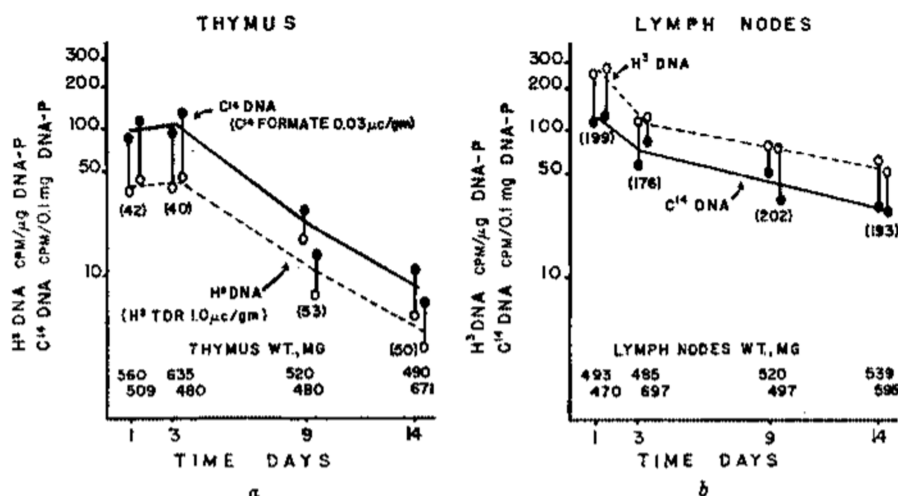


TEXT-FIG. 8 *a*. Relationship between thymus weight and H^3DNA specific activity in rats partially hepatectomized 5 days before H^3Tdr and sacrificed between 48 hours and 10 days after isotope administration.



TEXT-FIG. 8 *b*. Relationship between thymus H^3DNA specific activity multiplied by thymus weight and mean grain count of large thymic progenitor lymphocytes in rats receiving varying dosages of H^3Tdr and subjected to varying degrees of operative stress prior to isotope administration.

H^3 determined, as shown in Text-figs. 9 *a* and 9 *b*. C^{14} specific activity in RNA and non-DNA phosphorus pools was comparable in both tissues and followed a curve similar to that shown for DNA. It will be noted that the slopes of isotope loss were similar for both isotopes in both tissues and the ratio of carbon to tritium labeling remained nearly constant. It is evident that the initial uptake of H^3Tdr into DNA, relative to the uptake of C^{14} formate into DNA, is less in the thymus as compared to the lymph nodes.



TEXT-FIGS. 9 *a* and 9 *b*. Loss of H^3DNA (after H^3Tdr administration) and C^{14} DNA (after C^{14} formate administration) from the thymus and lymph nodes of normal rats. The figures in parentheses refer to the ratio of H^3DNA CPM to C^{14} DNA CPM (mean of 2 animals at each time period).

DISCUSSION

The results indicate two distinct patterns of initial uptake of H^3Tdr by the lymphatic tissue of normal rats and the subsequent loss of H^3DNA. The lymphatic tissue of the thymus cortex shows a very uniform pattern of a high percentage of large thymocytes which incorporate some H^3Tdr, albeit in small amounts, and a sharp decline in mean grain count during the 24 hour period following isotope administration. These findings are compatible with the proliferative activity of the thymus cortex as estimated by mitotic index and DNA turnover with other isotopes (22–25, 29, 30).

The low initial uptake of H^3Tdr per thymus cell is not explainable by a longer DNA synthetic period for these cells. The *in vitro* data and the marked alteration in H^3Tdr uptake after operative stress suggest that the lower uptake is not due to diminished availability of the isotope because of circulatory factors.

Potter and Nygaard (13, 31) have found that the rat thymus contains larger pools of thymine nucleotides than spleen. These pools did not become labeled after administration of isotopic thymidine and appeared to have a slow turnover rate. Non-DNA H^3 could not be detected in the present studies indicating that the H^3 label, once in DNA, did not appear to recycle through acid-soluble pools. Potter and Nygaard did not demonstrate precursor-product relationships between Tdr, TMP, TDP, and DNA-T, although administered labeled Tdr moved rapidly into new DNA. They concluded that the acid-soluble pools of thymine nucleotides were derived from some other metabolic pathway, probably from deoxycytidine monophosphate (dCMP) by means of dCMP deaminase as described by Maley and Maley (32).

In a correlative biochemical and autoradiographic study, Sugino, Frenkel, Bishop, and Potter described the changes which occur in the thymus recovering from whole body x-irradiation, which depletes the thymus of small cells (13, 33, 34). They showed a marked increase in uptake of labeled Tdr, administered 2 days after x-ray, by large precursors which they described as reticular cells and large primitive monocytoid lymphoblasts. This is similar to the increased uptake of H^3Tdr by large cells in rats previously stressed, as described here. The loss of thymus lymphocytes following irradiation was associated with enzymatic changes suggesting that the large progenitor cells were unable to synthesize thymidylic acid from dCMP and dUMP, depending upon donation of thymidine (or thymine nucleotides) from small lymphocytes. These authors' findings strongly suggest a feeding of thymidine or thymine nucleotides from small lymphocytes to progenitor cells and provide a possible explanation for the low uptake of H^3Tdr by normal thymus progenitor cells.

The transfer of labeled nuclear constituents from small thymus lymphocytes to larger proliferating thymus cells is suggested by the finding that the serial dilution of grain count over the large thymus lymphocytes with successive divisions followed a biphasic curve with two exponential components of differing half-times and by the fact that, after the appearance of H^3DNA in small cells, the subsequent loss of H^3DNA from the large progenitor cells occurs at the same rate as the loss of labeled DNA from the thymus (Text-fig. 8 *b*). Since the bulk of labeled DNA is in small cells after the first few divisions of labeled progenitor cells, it would appear that the H^3 label in the DNA of small thymus lymphocytes is reentering the DNA of the large progenitor thymocytes. Whether this apparent reutilization of DNA label can be related to the low initial uptake of H^3Tdr by progenitor cells in the thymus remains to be proved. The inability to detect non-DNA pools of labeled compounds after repeated doses of H^3Tdr and the similarity between the kinetics of DNA label loss with C^{14} formate and H^3Tdr weigh against the formation of labeled precursor pools from breakdown of H^3DNA.

The striking increase in uptake of H^3Tdr by thymic progenitor cells after depletion of the tissue content of small cells is different from the change observed when a tissue with a slow rate of growth, *e.g.*, the liver, is stimulated to replace lost tissue (35). Here, large numbers of hepatic cells which were not in

the process of cell division before partial hepatectomy can be observed to go into DNA synthesis. The significant increase in uptake of H^3Tdr involves the increased number of liver cells which will incorporate the label in contrast to the very few cells which would do so prior to hepatectomy; the mean grain count per labeled cell is not significantly altered. The removal of liver tissue activates enzymes concerned with DNA synthesis in cells which were not in DNA synthesis prior to the operation. The thymus, on the other hand, has a high mitotic index and DNA turnover rate in the resting state and contains all the enzymes for active DNA synthesis (31, 33, 34). The sequence of enzymatic changes which occurs in the regenerating liver prior to increased DNA synthesis does not develop during the recovery of the thymus from depletion by whole body x-ray (13, 31, 33, 34). There is a marked increase in the uptake of H^3Tdr by each thymus progenitor cell in DNA synthesis when the small cell population is depleted. This is presumably because some of the constituents for DNA synthesis are normally transferred from small cells to large.

The pattern of H^3DNA loss from the thymus following H^3Tdr administration is also quite different from that of peripheral lymph nodes and spleen. The apparent retention of total thymus H^3DNA for some 3 days, despite rapid dilution of the grains over progenitor cells, could mean that the labeled small cells, arising from divisions of these progenitors, do not leave the tissue in significant numbers for some 3 days. It could also mean that the H^3DNA which is lost from the thymus by the loss of labeled cells is replaced by labeled cells moving into the thymus. Thymus H^3DNA and H^3 content after 3 injections of H^3Tdr of 0.5 μc/gm at 24-hour intervals increased about five times above the values after a single injection. This suggests the accumulation of H^3DNA in the thymus over the 3 day period and would be consistent with the movement of labeled cells from the periphery into the thymus. Highly labeled small lymphocytes, such as those present in lymph nodes, blood, and most other tissues of the body, were rarely observed in the thymus cortex at any time after single or multiple doses of H^3Tdr. If labeled cells move into the normal thymus they must not be of this variety. However, a large population of small lymphocytes exhibiting low grain counts after H^3Tdr and rapid turnover has been described in the normal bone marrow (36, 37), and the present findings indicate a significant percentage of such cells in the germinal centers of lymphoid follicles in lymph nodes and spleen. This indicates that the metabolic factors leading to this pattern of H^3Tdr uptake in the thymus are not peculiar for that organ. Entry of this type of labeled lymphocyte into the normal thymus would not be detected by the techniques used.

The progressive loss of H^3DNA from the thymus, after the 3 day plateau of retained activity, follows a single exponential decline with a half-time of less than 3 days. This is compatible with the previously mentioned evidence of rapid turnover of normal rat thymus DNA. If cells containing H^3DNA move into the

thymus to explain the 3 day plateau of retained activity, as discussed previously, these labeled cells must have a very brief sojourn in the thymus.

The interpretation of the H^3DNA kinetic data for peripheral lymph nodes and spleen is complicated by the mixed cell populations in these areas. The spleen of the rat contains variable amounts of erythropoietic, myelopoietic, and megakaryocytic tissue as well as lymphatic and reticuloendothelial elements. The lymph nodes and thymus contain such relatively small amounts of replicating non-lymphoid tissue that the isotope kinetics probably reflect the label in lymphocytes. The similarity of the curves for H^3DNA and H^3 loss from the spleen and lymph nodes suggests that the bulk of splenic radioactivity is contained in its lymphoid tissue. Nevertheless, the course of DNA label dilution with time in lymph nodes and spleen follows a complex curve with at least two components. Furthermore, the results of autoradiographic analysis of lymphoblasts in these organs reveal two categories of large lymphocytes which incorporate H^3Tdr (Text-fig. 3 and fig. 1). Correlation of autoradiographic findings from imprint and section material indicates that the large lymphocytes in the germinal centers of lymphoid follicles have a low mean grain count. Similar findings have been made by others (27, 28).

The dilution of the DNA label in lymphocytic progenitor cells with successive divisions should reflect the rate of new DNA formation in this tissue, as discussed in the introduction to this paper. The high percentage of large lymphocytes which incorporate the label and the reduction of mean grain count to one-third of the initial value after 24 hours indicate a rapid generation or replication time for these cells. However, after 24 hours the rate of label dilution slows and appears to approximate the loss of radioactivity from the total lymph node. This finding is similar to the observations of Alpen *et al.* (14), who resolved the grain dilution in lymphoblasts into a two component curve and presented the reasons for considering this as evidence for reutilization of DNA label.

The correlation between mean grain count of the large lymphocytes and total lymph node H^3DNA, after the first 24 hours, is of considerable interest. The total H^3DNA in the lymph nodes is comprised of that in dividing cells, here represented by large lymphoblasts, and that which enters the small lymphocytes by division of progenitor cells (large or medium). The small cells, each of which contains the same amount of DNA as the interphase progenitor cells, outnumber the progenitor cells and contain about 80 per cent of the total DNA. Therefore, the DNA of small cells is the chief determinant of DNA specific activity after H^3Tdr administration. One hour after a single injection virtually all of the H^3 is in the DNA of progenitor cells (large and medium lymphocytes). After a few divisions of the progenitor population the H^3 content of the large lymphocytes contributes very little to the level of lymph node H^3DNA specific activity or H^3 content. These values then reflect the H^3 content of the numer-

ically superior population of small lymphocytes. The gradual loss of DNA label from the progenitor cells at a rate which approximates the loss from the entire bulk of small lymphocytes suggests that the DNA label in small lymphocytes reenters the progenitor lymphocytes.

Two component curves have been described for the dilution of DNA label in peripheral blood lymphocytes in humans (8–10), dogs (38), and rats (29, 39, 40). The finding has been generally interpreted as indicating two populations of lymphocytes in the circulating blood, one with a short life span and one with a very long life span. The present findings of varying rates of DNA turnover in different parts of the lymphatic system may be consistent with this view. Certainly the experiments of Little *et al.* (41) indicate a very long time for total lymphocyte DNA turnover. However, the present findings indicate that reutilization of DNA may occur in lymphatic tissue, as proposed by Hamilton (8) and Alpen *et al.* (14).

The apparent reentry of H^3DNA from small lymphocytes into the actively proliferating lymphoblasts renders very difficult the interpretation of this type of labeling data in terms of cytokinetics. Furthermore, the initial slope of loss of H^3DNA from lymph nodes may be influenced by the time required for equilibration to occur between labeled cells leaving and reentering the lymph nodes (20). These factors prohibit any assignment of "life span" to lymphocytes as estimated by DNA labeling techniques. It may be misleading to describe "short-lived" and "long-lived" populations of peripheral small lymphocytes. The actual fate of these cells is unknown. Nevertheless, there are at least two types of peripheral lymph node lymphocytes exhibiting differences in H^3Tdr labeling and DNA turnover. The turnover of DNA in the lymphocytes of the thymus cortex, bone marrow (36, 37), and in germinal centers (27, 28) of lymphoid follicles is rapid, and mitotic cells are most numerous in these areas. These findings point to a rapid rate of new cell formation. This is in contrast to the very slow rate of DNA turnover in the small lymphocytes formed in the mantle zones around lymphoid germinal centers and in the medullary cords and pulp of lymph nodes and spleen. The latter type of cell comprises the majority of peripheral lymphocytes.

Various mechanisms for the reentry of DNA from small lymphocytes into the nuclei of proliferating lymphoblasts have been proposed. These include phagocytosis of nuclear debris by reticuloendothelial elements capable of differentiating into lymphocytes (2, 39), pinocytosis or some similar process (4) and cellular transformation (2, 7). In view of the convincing demonstrations by others that small lymphocytes from the peripheral blood or thoracic duct are capable of reassuming the appearance and mitotic activity of lymphoblasts both *in vivo* (7, 21, 42, 43) and *in vitro* (44), it would seem that this mechanism may explain the findings in peripheral lymph nodes and spleen. The resumption of proliferative activity by small cells undergoing antigenic exposure in various parts of the far flung peripheral lymphatic tissue of these rats living with the usual laboratory microbial flora could explain the apparent reentry of H^3DNA from small cells into large progenitor cells without invoking actual transfer of nuclear material between cells.

The possibility that small lymphocytes may assume the appearance and

mitotic behavior of lymphoblasts might explain several enigmatic observations concerning the response of the lymphatic tissue to injury. Nygaard and Potter employed serial determination of C^{14}DNA specific activity in the lymphatic tissues of rats receiving labeled thymidine and then subjected to 400 roentgens whole body irradiation (13). They found little or no alteration from normal in the rate of DNA label loss from the tissue remaining, although the volume of lymphatic tissue was markedly reduced. They concluded that the x-rays must have destroyed small cells and large progenitors in roughly equal numbers. Alpen, Cooper, and Barkley, in a similar study (14), found that the rate of dilution of labeled DNA in progenitor cells of lymph nodes was unaffected by 300 r whole body irradiation. The findings reported here in a small number of rats subjected to operative stress with resulting involution and recovery of lymphatic tissue are similar. Recovery from this type of acute depletion results in a shift in cell population toward more immature larger lymphocytes The surprising failure of these severe perturbations to cause profound alterations in the rate of DNA renewal and turnover, as reflected by the rate of DNA label loss could be explained if the small lymphocyte has the same proliferative capability as the larger cell. If this were the case removal of lymphocytes would remove cells with the ability to divide regardless of whether they are large and "immature" or small and "differentiated." In terms of DNA labeling the present findings show that it is not possible to draw a clear line between small, morphologically mature cells and large progenitor cells capable of mitosis. The concept of the small lymphocyte as a progenitor cell, discussed extensively by Yoffey (45), originates from the early observations of Maximow (46), Jordan (47), and others.

The absence of recirculation to the thymus of small lymphocytes formed in the mantle zones around germinal centers may have significance with respect to certain views of the participation of the thymus in immune reactions (48–50). There now appears to be ample evidence that thymus small lymphocytes have a role in immunological events that differs from that of lymph node or spleen small lymphocytes. Thymus cells do not have the potency of peripheral lymphocytes in transferring adoptive immunity or initiating the graft versus host reaction when given to tolerant recipients (51, 52). The metabolic and kinetic differences between "thymus-like" lymphocytes and peripheral lymphocytes may reflect basic differences in physiology and function between various types of lymphoid cells.

SUMMARY AND CONCLUSIONS

Cytokinetic data are presented, employing quantitation of H^3DNA in the lymphatic tissues of normal rats serially sacrificed after H^3Tdr administration. A marked difference in the patterns of initial labeling and label loss was observed between the thymus and peripheral lymphatic tissue.

The data are compatible with other indications of rapid cell renewal in the thymus. There is suppression of initial uptake of H^3Tdr into the DNA of each large lymphocytic progenitor cell in the thymus, apparently because of a feedback of thymidine containing material from small lymphocytes in the thymus. Depletion of the thymus of small cells, as by operative stress or whole body x-ray, leads to a marked increase in the uptake of H^3Tdr into the DNA of large thymocytes. This finding, which is in agreement with the previous findings of Sugino *et al.* (33, 34) suggesting transfer of thymine nucleotides from small thymus lymphocytes to precursor cells, may or may not be related to the apparent transfer of DNA label between thymic cells. The evidence for the latter consists of the curvilinear dilution of the DNA label in the thymus proliferating cell population and the relationship between the rate of DNA label dilution in large cells and the H^3DNA in the small cells in the thymus.

After the DNA label in progenitor cells in the thymus and lymph nodes has entered the small cell population, the subsequent dilution of grains in these dividing cells follows the same slope as the loss of radioactivity from the entire lymph node. There is a long retention of some H^3DNA label in the dividing lymph node cell population. This suggests that the loss of radioactivity from the dividing cells and from the small cell population as a whole occurs equally. This pattern prevails regardless of whether the percentage of large and small cells is altered experimentally. These findings can be explained by an interchange of the DNA nuclear label between small lymphocytes and large lymphocytes. This could occur by some process such as phagocytosis or pinocytosis, or by transformation of the small lymphocyte into a large, dividing cell. The data fit best with the latter possibility. All or any of these mechanisms would lead to an equilibration of the DNA label between large and small cells. This finding prevents the assignment of a finite life span to lymphocytes on the basis of DNA labeling kinetics. Nevertheless, there appear to be at least two different types of lymphocytes. One, the "thymus-type" lymphocyte, is found in the thymus cortex, bone marrow and germinal centers of lymphoid follicles. The other type, found abundantly in the widespread peripheral lymphatic tissue, shows a very prolonged retention of DNA label and is believed to be the recirculating, "immunologically committed" cells described by others. These cells do not appear to enter the thymus cortex.

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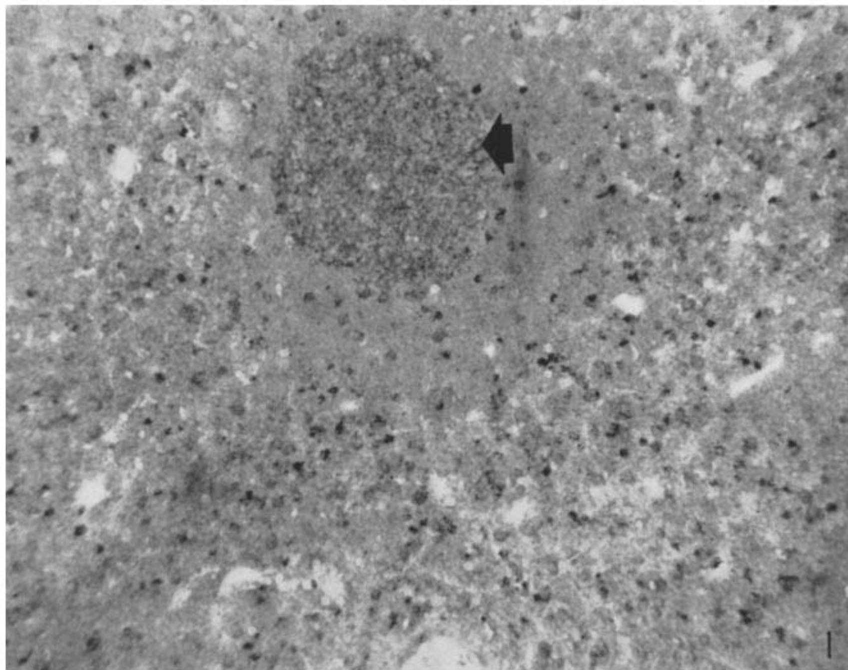
EXPLANATION OF PLATES

Plate 39

Fig. 1. Germinal center in mesenteric lymph node of normal rat sacrificed 1 hour after H^3Tdr, 0.5 μc/gm, showing highly labeled cells (black dots) in the periphery of the follicle and in the pulp of the node. The germinal center (arrow) shows large numbers of less highly labeled lymphocytes. $\times$ 168.

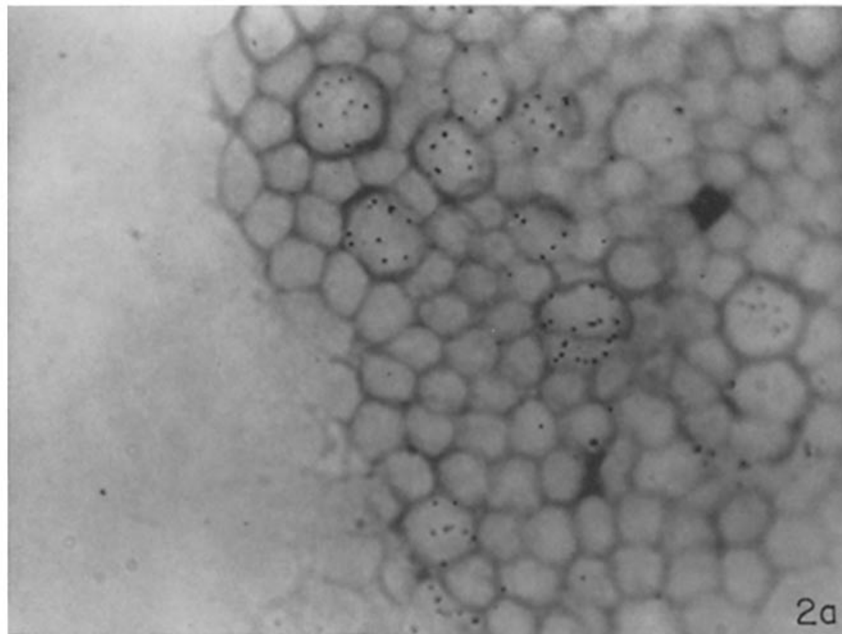


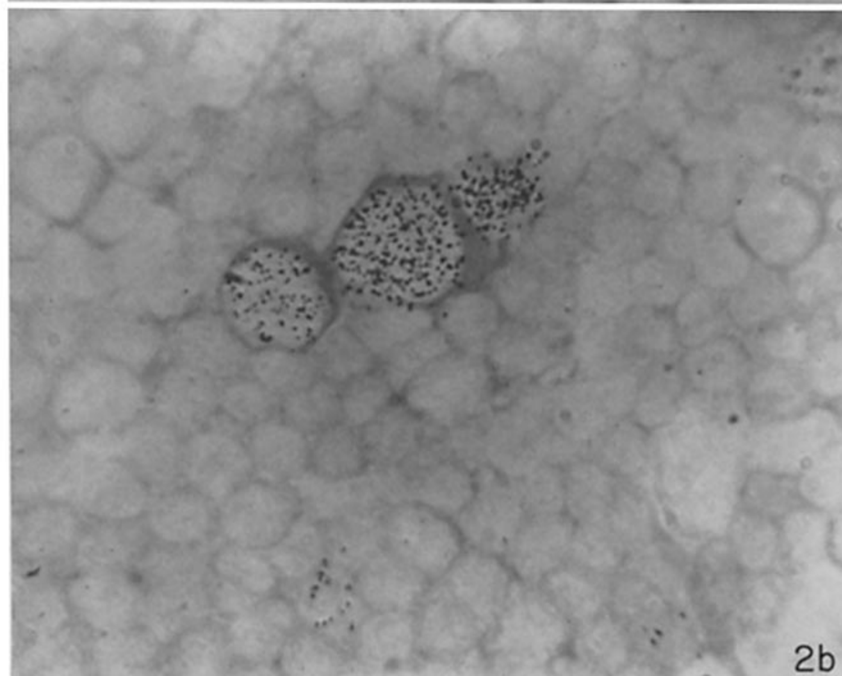
(Craddock *et al.*: Proliferative activity of lymphatic tissues)

PLATE 40

FIG. 2 *a*. Imprint showing labeled large lymphoid cells in the thymus cortex of a normal rat sacrificed 1 hour after 1.0 μc/gm of H^3Tdr. × 1400.

FIG. 2 *b*. Highly labeled large and medium lymphocytes in the mesenteric lymph node of the same rat. × 1400.

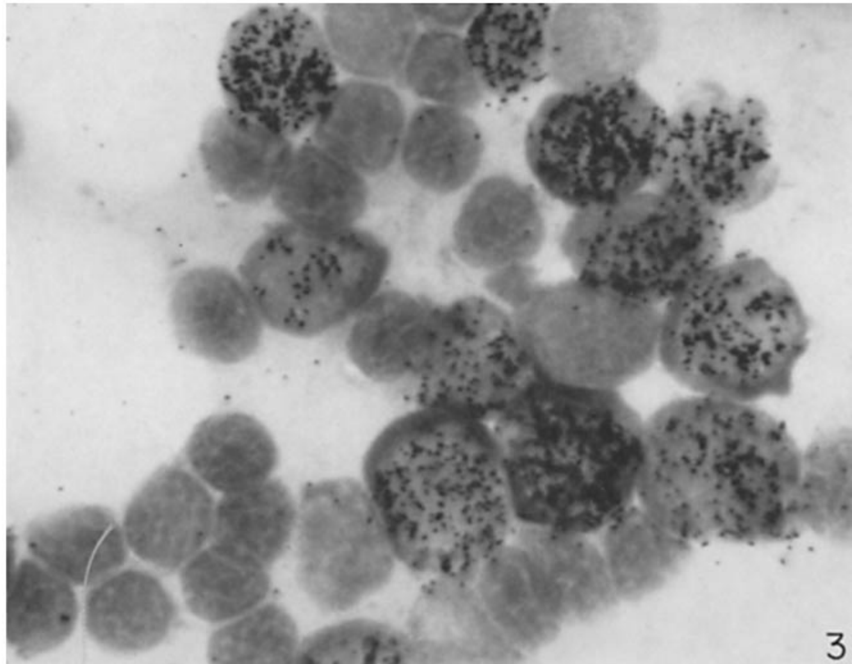




(Craddock *et al.*: Proliferative activity of lymphatic tissues)

Plate 41

Fig. 3. Labeled large lymphoid progenitor cells in the thymus cortex of a rat subjected to 50 per cent hepatectomy 3 days before administration of 0.5 $\mu c/gm$ of H^3Tdr and sacrificed 1 hour later. $\times$ 1400.



(Craddock *et al.*: Proliferative activity of lymphatic tissues)