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The Reutilization of Thymocytic and Lymphocytic Desoxyribonucleic Acid

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THE REUTILIZATION OF THYMOCYTIC AND LYMPHOCYTIC DESOXYRIBONUCLEIC ACID

by

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LIFE OF AUTHOR

Kenneth Robert Goljan was born in Chicago, Illinois on March 16, 1937.

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CHAPTER I

INTRODUCTION

The continued synthesis of nucleic acids is essential to the maintenance of a homeostatic state within a mammal. It is generally believed that nucleic acids are synthesized from their fundamental components; a free metabolic pool of nucleic acids has not been described in the literature.

The purpose of this work is to consider the hypothesis that, aside from their immunologic function (Burnet and Ferrer 1949, Burnet 1962, Jankovic 1962, Auerbach 1963), the ultimate fate of lymphocytes and thymocytes is to release their nucleic acid upon cytolysis, for reutilization by other lymphocytes and thymocytes, and other cells undergoing chromosomal replication. This release and reutilization of lymphocytic and thymocytic nucleic acid - after the usefulness of lymphocytes and thymocytes as antibody producers and carriers is completed and their life span terminated - represents a system of preparing and supplying trephones (nutritive substances supplied by trephocytes which are feeding or nutritive cells - in this case lymphocytes and thymocytes) to those areas needing im-

mediately available sources of readily utilizable desoxyribonucleic acid. This desoxyribonucleic acid is necessary for normal growth and maintenance, as well as, pathological growth processes, and growth following degenerative processes such as regeneration and fibrosis.

CHAPTER II

REVIEW OF THE LITERATURE

The basic idea of trephocytes (feeding or nutritive cells) supplying trephones (nutritive substances supplied by trephocytes) is not of recent origin. As early as 1715, Dionysius felt that the thymus secreted a fluid which was nutritive for the newborn. Later in 1845, Goodsir and Goodsir described minute cellular parts, which were nuclei, which they concluded were centers of nutrition distributing nutritive materials to each organ. Carell (1924) concluded from his observations that leukocytes carry "embryonic growth-promoting substances as trephones" which he found essential to the growth and maintenance of tissue cultures. From Carells' work, Dustin (1933) reasoned that the storage and liberation of nucleoprotein was the prime function of thymocytes.

In a more current frame of reference, the problem regarding the fate of lymphocytes and thymocytes has been challenging - especially when one considers the rapid rate of turnover of these cells. The only tissue examined by v.Euler and Hevesy (1942) in which the nucleic acid turnover was found to be of the same magnitude as that in the thymus was the rapidly growing

Jensen sarcoma. Andreasen and Ottesen (1945) observed that the rate of nucleic acid renewal was five to six percent in three hours in the thymus gland as compared to the lymph nodes and spleen at one to two percent in the same time. They concluded that, compared to the high thymic activity, the other lymphoid organs possessed only a slight lymphocytopoietic function. The percentile ratio of new-formed nucleic acid was determined by Andreasen and Ottesen (1944) twenty-four hours after the administration of 2μc of P^{32} subcutaneously in albino rats. The percentile ratios were found to be as follows: thymus 31.8, spleen 9.2, Peyers patches 7.3, intestinal lymph nodes 4.9, subcutaneous and lung lymph nodes 4.7. These percentile ratios were determined on three month old rats, at a time when the period of growth of lymphoid tissue is stabilized and regressive changes have not yet appeared. Therefore, Andreasen and Ottesen concluded that the nucleic acid turnover was not due to processes of growth increasing the volume of each respective organ, but rather was attributable to compensatory growth in order to maintain a continual lymphocytic output to the lymph and blood stream. Gowans (1957a) was able to demonstrate that the daily output of lymphocytes, obtained by means of cannulating the thoracic duct of unanesthetized rats, was sufficient to replace all the lymphocytes in the blood about eleven times daily. The greatest mitotic activity was found by Andreasen and Christensen

(1949), in rats, to be in the thymus gland in which there were four to six times as many mitotic figures than in lymph nodes. From these observations, one is led to the obvious conclusion that the rate of cell turnover in the thymus gland and lymphoid tissue is remarkably high.

Hamilton (1954, 1956) utilizing adenine-8-C^{14} was able to demonstrate in a patient suffering from chronic lymphocytic leukemia, that human desoxyribonucleic and ribonucleic acid activity declined in two phases. The initial more rapid phase was approximately of two months duration, during which time the decline in ribonucleic acid activity greatly exceeded that of desoxyribonucleic acid. During the later phase, the decline in activity of ribonucleic and desoxyribonucleic acid was more nearly parallel, and the duration of the phase was three hundred days; after which time the desoxyribonucleic acid was still at more than one-third of the peak activity. From the gradual decline in desoxyribonucleic acid activity, it was concluded that either leukemic lymphocytes have an unusually long life span, or the lymphocytes in question can utilize specifically, with great efficiency, large fragments of nucleoprotein of their progenitors. A two phase system similar to that described by Hamilton was observed by Ottesen (1954), dealing with normal human subjects - but the phases represented a shorter life span for lymphocytes than indicated by Hamilton. Ottesen administered

200μc of P^{32} intravenously and found twenty-four percent of the lymphocytes had a mean age of from three to four days while seventy-eight percent had a mean age of from one hundred to two hundred days. Albino rats were continually infused by Little, et al (1962) with tritiated thymidine - the mean age of large lymphocytes in the peripheral blood was found to be sixty days while for small lymphocytes there was an age in excess of one hundred days. Yoffey (1933, 1950) estimated the average life span of lymphocytes to be approximately four hours; however, he also observed (1950) that lymphocytes survived for at least twenty-six days in modified Clark-Sanderson windows in the ears of rabbits. McCutcheon (1953) concluded that lymphocytes are renewed approximately three times daily or every eight hours. These observations on the life span of lymphocytes seem to indicate a confused state of affairs. However, one must speculate that the evidently long life spans are due to one of three alternatives; either to long lived lymphocytes, or lymphocytes which have reutilized radioactive desoxyribonucleic acid fragments, or both. The second alternative appears most plausible in view of the rapid rate of cell turnover indicated earlier and from Trowell's (1957) observations.

Trowell (1957) observed that reticulum cells of the albino rat lymph node differentiated, in vitro, into large lymphocytes - some of these differentiating cells still contained remnants of

ingested small lymphocytes. This observation suggests that the nuclear material of old lymphocytes may be directly reutilized in the formation of new lymphocytes. It is estimated by Kindred (1942) that thirty-five percent of the lymphocytes produced are eventually disposed of in this way. The observations made by Trowell further compliment Hamilton's (1954, 1956) and Ottesen's (1954) observations on an apparently long persistent lymphocytic radiolabel, which in this case suggests that the desoxyribonucleic acid of old lymphocytes may be specifically reutilized, without degradation, in the genesis of new lymphocytes. DeBruyn (1948) observed that the germinal centers which contained the greatest number of phagocytosed dead lymphocytes were also the most active areas of lymphopoiesis. He indicated that mitotic activity is frequently accompanied by necrobiotic phenomena in developmental processes, and that the lymphocytic population may be controlled to some extent by this process; which may constitute a self regulating "feed-back" mechanism. Reticular cells in the spleen, adjacent to fragments of lymphocytes, were observed by Hill (1959), to show a gradual rise in cytoplasmic basophilia, and the nuclei and nucleoli became enlarged. There appears to be a close time relation between the dissolution of lymphocytes and the differentiation of reticular cells; eventually, the enlarged pyroninophilic reticular cells develope into more differentiated cells - plasma cells and large and medium

sized lymphocytes. The observation was also made by Hill that in cases of lymphocytic "deprivation" a "dedifferentiation" to a more immature stage was assumed by the differentiating reticular cells. Medawar (1957) cites several observations, using time-lapse cinematography, demonstrating lymphocytes often moving towards and adhearing to malignant cells, megakaryocytes, and to cells of all kinds which eventually went into mitosis. He felt this was an indication that perhaps the lymphocytes in question may be in the process of contributing their desoxyribonucleic acid.

The liver will degrade approximately sixty-three percent of thymidine and approximately eighty-seven percent of thymine within two hours after the intraperitoneal administration of radiolabeled thymidine and thymine (Potter 1959). Dancis and Balis (1954) utilized a pregnant rat, tumor-bearing mice, and parabiotic rats in which the host animals were labeled with adenine-8-C^{14} (sp. act. 68,500 c.p.m. per μ M). They suggest that purines liberated into blood, as a result of nucleic acid catabolism, are not reutilized for nucleotide synthesis to any great extent. Further, Cronkite, et al (1959) felt that desoxyribonucleic acid and its metabolites were not passed on from one cell to another. Cronkite, et al based their assumption on the disappearance of intravenously administered tritiated thymidine from the blood in a matter of minutes. They concluded

that tritiated thymidine was incorporated into desoxyribonucleic acid and the labeled thymidine not so incorporated was catabolized. Though the purines and pyrimidines may not be readily utilizable after a period of time when liberated in the blood and carried to the liver, it appears that they may certainly be readily available upon cytolysis of cells in the relative proximity of other cells undergoing chromosomal replication; in which case, the free desoxyribonucleic acid would probably not reach the liver. The reutilization of desoxyribonucleic acid is alluded to in the observations made by Kindred (1942), Hill (1959), Kelsall and Crabb (1959), Hill and Drasil (1960), Bryant (1962), and Rieke (1962).

Gowans (1959b) observed gross radioactivity within twelve hours after the infusion of P^{32} labeled lymphocytes, mainly in the deep cervical, mesenteric, submaxillary, and thoracic lymph nodes, and spleen. Observations of radioactivity in bone marrow, liver, lung, ileum (including Peyers patches) and ascending colon were fairly low, with the kidney exhibiting practically no radioactivity and the thymus indicating no activity. However, the thoracic duct was cannulated throughout the experiment and a significant stress was obviously placed on the restrained experimental animals; it has been demonstrated (Reinhardt and Yoffey 1956 and Andreasen 1943) that animals maintained under stressful conditions will experience a depression of mitotic activity.

This may have accounted for the absence of a radiolabel in the thymus gland (Yoffey 1960). Shorter and Bollman (1960) contradict, to some extent, the data observed by Gowans (1959b) in that the thymus gland, liver, and intestine demonstrated a higher degree of activity than that observed by Gowans. Fichtelius (1953) studied the fate of radiolabeled thymocytes and found a much higher degree of label in all tissues studied than that observed by Gowans (1959b) and Shorter and Bollman (1960). The conclusions derived from the above experiments were founded on the gross radioactivity of a tissue, with the assumption that this radioactivity was due to the presence of labeled lymphocytes as such. No histologic examinations were made to determine if the radioactivity observed was due to the reutilization of radiolabeled desoxyribonucleic acid or radiolabeled lymphocytes and thymocytes as such.

With the advent of tritiated thymidine as a specific desoxyribonucleic acid precursor (Friedkin, et al 1956, Amano, et al 1959, and Cronkite, et al 1958, 1959), some significant studies have been made on the reutilization of lymphocytic and thymocytic desoxyribonucleic acid utilizing autoradiographic techniques. Fichtelius and Diderholm (1961) have been able to demonstrate a greater than normal number of significantly labeled "round cells", fibroblasts, and epidermal cells at wound sites in previously radiolabeled animals. These observations were explain-

ed by the hypothesis of desoxyribonucleic acid reutilization. These observations appear to be among the first such definite demonstrations. However, no evidence was obtained to clarify the question as to whether labeled desoxyribonucleic acid precursors arose locally from the breakdown of labeled leukocytes acting as trephocytes of desoxyribonucleic acid or whether the label arose as a result of the breakdown of labeled cells in other areas and was carried to the injury site.

Bryant (1962) noted the labeling of regenerating liver cells after the injection of isologous lymphocytes and thymocytes labeled with tritiated thymidine. He considers that liver cells, regenerating and consequently involved in desoxyribonucleic acid synthesis, became labeled as the result of a local breakdown of radiolabeled lymphocytes acting as trephocytes of desoxyribonucleic acid. Hill and Drasil (1960) injecting P^{32} labeled thymocytes in lethally irradiated mice were able to demonstrate labeling of the thymus, spleen, bone marrow, liver, kidney, and duodenum cells. However, more than half of the radioactivity of the injected cells was not in fully polymerized desoxyribonucleic acid, but rather in acid-soluble compounds. Therefore, the possibility exists that the labeling found in the recipient animals may have originated from compounds other than desoxyribonucleic acid. In contrast to the work of Dancis and Balis (1954), mentioned earlier, in which they could not demonstrate the re-

utilization of desoxyribonucleic acid by implanting a sarcoma in mice which were labeled with adenine-8-C^{14}; Rieke (1962) demonstrated the labeling of sarcoma cells transplanted to host animals which were previously labeled with tritiated thymidine. He also demonstrated the labeling of sarcoma cells by the administration of tritiated thymidine labeled lymphocytes into tumor bearing animals. Rieke attributes the lack of radiolabeling in Dancis and Balis' experiments to the use of a radiolabeled compound too low in activity. Rieke made a very significant observation, in that he was able to demonstrate that more cells may be labeled with a heavier label when polymerized tritiated thymidine labeled desoxyribonucleic acid was administered to recipient animals as compared to radiolabeled desoxyribonucleic acid which was ninty-five percent depolymerized or hydrolyzed. These observations appear to demonstrate that desoxyribonucleic acid and possibly its metabolites can be reutilized; which appears to place some doubt on the finality of the conclusions made by Cronkite, et al (1959), Potter (1959), and Dancis and Balis (1954) with regard to non-reutilization of desoxyribonucleic acid and its metabolites.

Bunting and Huston (1921) concluded that, in the albino rat, the great number of lymphocytes and thymocytes produced daily are eliminated from the intestinal tract. Though this observation may be true to some extent, it cannot account for the

disappearance of the great number of lymphocytes from the blood daily. However, it is normal for rabbits and rodents to practice coprophagy (Barki, et al 1949), so that actually the trephocytic concept is still maintained in this instance. Kelsall and Crabb (1959) state that by practicing coprophagy, a natural source of increased amounts of B-vitamins, nucleic acids, and mucopolysaccharides derived from bacterial action and the desintegration of bacteria in the stomach and intestines is made available to rodents.

Among the major proponents of the concept of the gross recirculation of lymphocytes are Gowans (1957, 1959a) and Mann and Higgins (1950) who, because there is a progressive decrease in thoracic duct lymphocytes, as determined by cannulation of the thoracic duct and an observation on the low rate of lymphocyte formation, propose essentially that the major percentage of lymphocytes must recirculate, and therefore, reutilization of desoxyribonucleic acid is untenable. However, the rate of lymphoid activity appears to be quite adequate as indicated earlier in the observations of v.Euler and Hevesy (1942), Andreasen and Ottesen (1944, 1945), Andreasen and Christensen (1949). Further, lymphopenia has been observed by Andreasen and Gottlieb (1947) to persist for several days as a result of operative procedures. Gowans, Mann, and Higgins' animals were restrained in a Bollman (1948) cage in which they did not gain weight or lose weight and were likely to be under stress. Loss of weight or lack of weight

gain were considered to have no effect upon the output of lymphocytes by Gowans (1957, 1959a) and Mann and Higgins (1950). However, Reinhardt and Yoffey (1956) and Andreasen (1943) have made several significant observations, especially the later investigator, on the profound depressing effects of stress and inanition on lymphocytic proliferation.

The migration of lymphocytes and thymocytes to various tissues appears to be variable, depending on the source of the cells in question. Radioactive phosphorous labeled thymocytes apparently have a predeliction for the spleen, while radiolabeled lymphocytes seemed to be concentrated in bone marrow (Fichtelius 1958a, 1958b) - both lymphocytic and thymocytic activity could be located in the liver. Diderholm and Fichtelius (1959) using P^{32} labeled cells, observed that some lymphocytes and much thymocytic activity was apparent perifollicularly in the spleen, though only lymphocytic activity was noted in the intestinal lymph nodes. Fichtelius and Diderholm concluded that for some unknown reason, thymocytes and lymphocytes are treated in different ways by the recipient animals.

By using the vital stain 3-6 diamino-10-methyl-acridinium hydochloride (acridine orange), which has an affinity for nucleoprotein, to label lymphocytes, Farr (1951) observed localization of labeled lymphocytes in bone marrow, thymus gland, mesenteric lymph nodes, appendix, Peyers patches, and spleen.

The further observation was made that those lymphocytes in the bone marrow appeared to be transformed into myelocytes; no erythroid precursors or macrophages were observed to be labeled, nor was a significant number of labeled lymphocytes present in the blood after ninety minutes. One may be inclined to feel that perhaps the labeled myelocytes seen were in fact cells which had taken up stain either released or still fixed to the nucleoprotein of cytolized lymphocytes - from the work of Rieke (1962) the latter alternative seems most plausable.

Andrew and Andrew (1949) claim that there is no evidence to indicate that lymphocytes which appear within the epidermis degenerate, but rather these cells are transformed into ordinary epithelial cells. However, Andreasen (1952) claims to have seen lymphocytes degenerating in the epidermis and states that this is one of the disposal areas for the lymphocytes of the body. Though no suggestion was made that the desoxyribonucleic acid of the cells, Andreasen observed to be degenerating in the epidermis, may be in the process of supplying nucleoprotein to some germinative cells of the epidermis, it is an interesting speculation.

The concept of small lymphocytes being cells capable of dedifferentiating into more immature and multipotential stem cells was first formulated in 1909 by Maximow and supported to a considerable extent by Bloom (1938), Yoffey and Drinker (1939) Kindred (1942), Farr (1951), and Dameshek (1963). However, doubt

is placed on the validity of the concept by the work of Ottesen (1954), Hamilton (1954, 1956), Trowell (1957), Medawar (1957), DeBruyn (1948), Hill (1949), Kelsall and Crabb (1959), Schorley and Berman (1960), Hill and Drasil (1960), Bryant (1962), and Rieke (1962). Rather, the weight of evidence appears to favor the hypothesis of reutilization as opposed to dedifferentiation.

CHAPTER III

MATERIALS AND METHOD

Six, twenty-one day old male albino rats of the Sprague Dawley strain were used. The animals were littermates and were maintained in wire bottom cages. The ears of all animals were notched in order to identify the animals (Billingham and Silvers 1961).

Two animals were selected as recipients and four animals were selected as donors. The donor animals received daily intraperitoneal injections of tritiated thymidine (sp. act. 1.9c/mM)[1] at the rate of 1μc per gram body weight, for eight days. The total administered dosage of tritiated thymidine was 0.80 mc for three donor animals and 0.70 mc for one donor animal, the difference in dosage being due to differences in total body weight.

After eight days of tritiated thymidine injections, one donor animal at a time was anesthetized on the ninth day by means of ethyl ether inhalation. The spleen and thymus gland were removed immediately by way of a mid-line incision through the skin, abdominal musculature, and sternum. A sample of the spleen and thymus, approximately one millimeter thick, was obtained, placed

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[1]Obtained from Schwarz Bioresearch Inc., Orangeburg, N. Y.

on a small square of paper appropriately labeled, and initially frozen for ten seconds in liquid isopentane which was chilled to approximatel -190°C. by means of liquid nitrogen. After a ten second interval in isopentane, the tissues were transferred to a plastic tube containing liquid nitrogen which was placed in a freeze-drying apparatus[2]. The tissue samples were maintained in the freeze-drying apparatus for fifty days at -30°C. under a constant high vacuum and dehydrated by means of phosphorous pentoxide. After obtaining samples of the spleen and thymus, as indicated above, the remainders of these organs were placed in separate flasks containing chilled Hanks solution and maintained at 20°C. in a refrigerator. One-half cubic centimeter of blood was withdrawn from the right ventricle of the heart into a heparinized syringe. Blood smears were prepared and the remainder of the blood was subjected to the freeze-drying procedure indicated above. Thin slices of the following tissues were obtained and subjected to the freeze-drying procedure: lung, testes, liver, kidney, tongue, oral mucosa, submandibular salivary gland, intestinal lymph nodes, bone marrow, and cervical lymph nodes.

After the four donor animals had been sacrificed and the necessary samples obtained and treated as indicated, the four thymus glands were macerated together through a monel metal screen

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[2]Hudson Bay Co., Model 4025 Freeze-Drying Apparatus

with one hundred and fifty grids per square inch (figure 1) with an angulated spatula according to the method of Krakower and Greenspon (1951). Buffered normal saline (pH 7.3) was added to the macerate to obtain a homogeneous cell suspension. Smears were made of the cell suspension and one-tenth cubic centimeter was subjected to the freeze-drying procedure. A cell count was made of the cell suspensions, utilizing a hemocytometer. The remainder of the thymus cell suspension (502,500 cells) was injected intraperitoneally into a recipient animal.

The same procedure was followed for the spleens of the four donor animals as was followed for the four thymus glands indicated above. However, the spleen cell suspension (19,305,000 cells) was injected intraperitoneally into a different recipient animal.

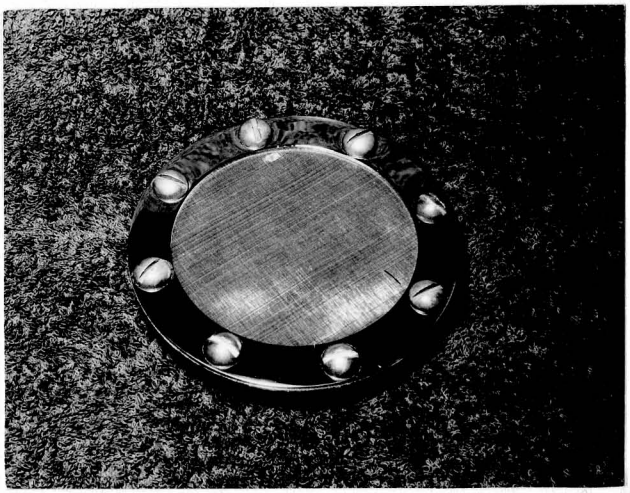

FIGURE 1 Monel metal screen and fram used to prepare thymus and spleen cell suspensions.

The two recipient animals were sacrificed seventy-two hours after receiving their respective injections of thymus and spleen cell suspensions. Tissue samples of lung, testes, liver, kidney, tongue, oral mucosa, submandibular salivary gland, thymus, spleen, intestinal lymph nodes, bone marrow, cervical lymph nodes, and blood were removed and subjected to the freeze-drying technique as indicated.

Smears prepared from all cell suspensions and blood samples were air dried and fixed in absolute methyl alcohol for forty-five minutes. All other tissue samples were freeze-dried as indicated, embedded in paraffin, and sectioned at three to six microns. Autoradiograms were prepared of both tissue sections and smears, using Kodak NTB3 liquid emulsion essentially according to the method of Joftes (1959). After the autoradiographic emulsions were exposed for four weeks, they were developed, washed, fixed, and stained with hematoxylin and eosin. Staining was deferred until after the development of the autoradiographic emulsion in order to eliminate leeching out of stain from the tissue sections.

Upon examination of the autoradiograms, those slides or areas which were obviously high in background grain counts were disregarded. All observations were made at one thousand magnifications using an oil immersion lens, and significantly labeled representative cells were recorded by means of photomicrographs.

The criteria upon which a cell was considered labeled was based essentially on the technique of Bryant (1962) which suggests that any nuclei with more than two grains could be considered a significantly labeled cell, provided the background was not greater than one silver grain per cell.

The total grain count in five representative acellular fields was recorded for each slide examined, and the total number of cells in five representative fields was also recorded. The background grain count was determined by dividing the average number of grains in a representative high power field by the average number of cells in a representative high power field. The background grain count per cell was then arbitrarily further increased by one hundred percent to compensate for chance false labeling of cells (Bryant 1962). Therefore, any cell having a grain count higher than this value could be considered a significantly labeled cell. The background grain counts on the slides selected for study were on the order of sixty to ninety grains per high power field; occasional autoradiograms contained as few as forty background grains per high power field. In those areas where the calculated grain count, at which a cell was considered labeled, was below one or two grains - the number was arbitrarily set at three grains to further obviate the chance of false labeling of cells. Cells having three grains per nucleus, with a one grain per cell background, may be regarded as significantly labeled according to Pelc (1959), Cronkite, et al (1959), and Bryant

(1962).

The percentage of radiolabeled cells in the thymus and spleen cell suspensions was determined by counting the number of radiolabeled cells per thousand cells.

Quantitative radiologic assays were performed on all samples obtained from the recipient animals and from one represetative donor animal. A Nuclear Chicago Liquid Scintillation 701 System was used in conductiong the assays. The efficiency of the system had been found to be eighteen percent. The glass sample vials[3] had a low potassium content in order to minimize the effect of natural radioactive potassium (K^{40}). Two milligram samples were weighed directly into the sample vials, using an analytical balance. All samples were brought into solution by means of one-half milliliter of a one molar solution of hyamine hydroxide in methanol[4] at 60°C. for forty-five minutes. After the sample solutions had cooled to room temperature, fifteen milliliters of a scintillating solution was added to each counting vial. The scintillator solution was composed of: (1) 2-5-diphenyloxazole (PPO)[5] 0.60 g., (2) p-bis-((2-5-(phenyloxazolyl))) benzene (POPOP)[5] 10 mg., and (3) toluene 500 ml. The data and gate voltages were set at 1225 volts and 1325 volts, respectively; channel 1 was set at Base to L_1 at eight volts. The room

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[3,4] Obtained from Nuclear Chicago Corp., DesPlaines, Ill.

[5] Obtained from Packard Instrument Co., LaGrange, Ill.

temperature during the counting procedure was 19°C. and the samples were chilled to 3°C. Ten one minute counts were made on each sample. To determine the internal quenching effect the sample solutions may have had, two-one-hundreds of a milliliter of standardized tritiated toluene[6], acting as an internal standard, was added to each counting vial and the samples were again recounted. The relative disintegration rate per minute (dpm) of the samples was determined according to the following equation (Hayes 1956, Davidson and Feigelson 1957) which takes into account internal quenching and internal standards:

$$\text{Disintegration rate of sample} = \frac{(\text{sample count rate}) - (\text{background count rate})}{\left(\begin{array}{c}\text{internal standard} \\ + \\ \text{sample count rate}\end{array}\right) - (\text{sample count rate}) \dfrac{\text{(internal standard)}}{\left(\begin{array}{l}\text{disintegration} \\ \text{rate}\end{array}\right)}}$$

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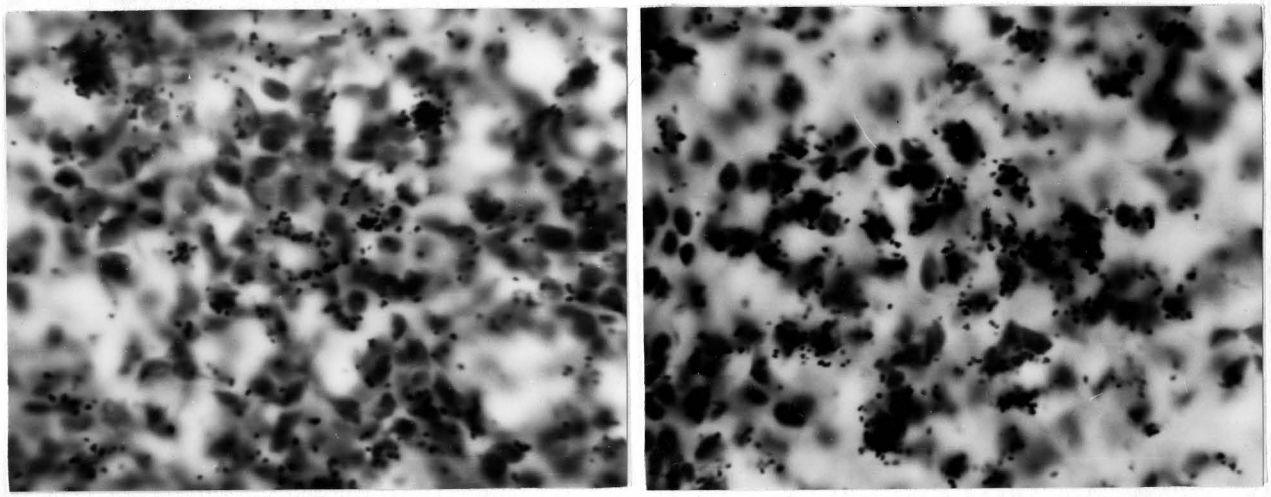
[6]"Standard Source Compound" obtained from Packard Instruments Company, LaGrange, Ill.

CHAPTER IV

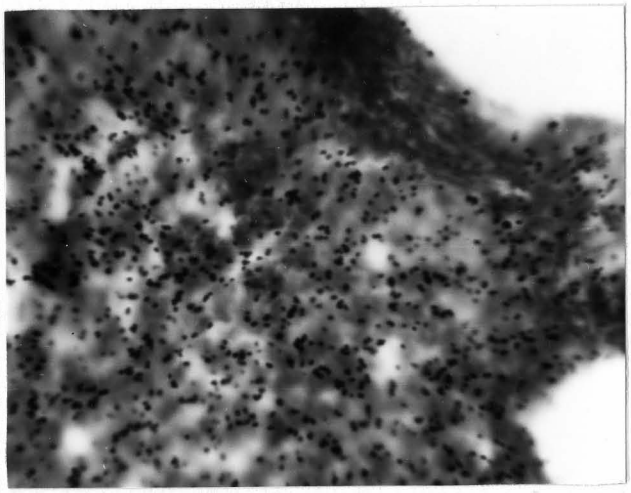
EXPERIMENTAL RESULTS

The extent of radiolabeling of donor animals receiving 1μc of tritiated thymidine (sp. act. 1.9 c/mM) per gram of body weight daily for eight days, is demonstrated by autoradiograms of some representative tissue sections in figure 2.

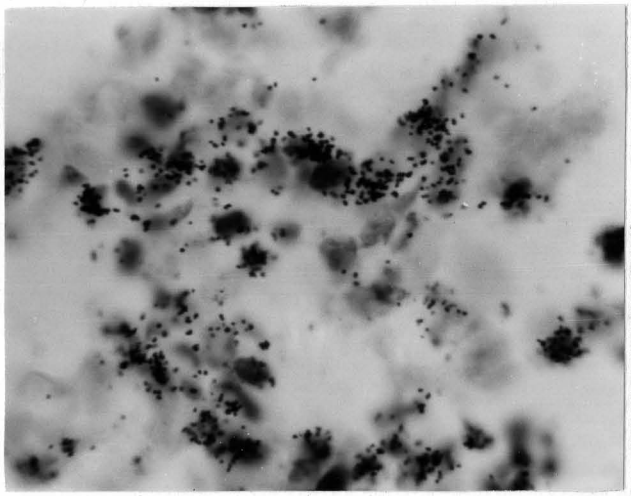


A. Thymus B. Spleen

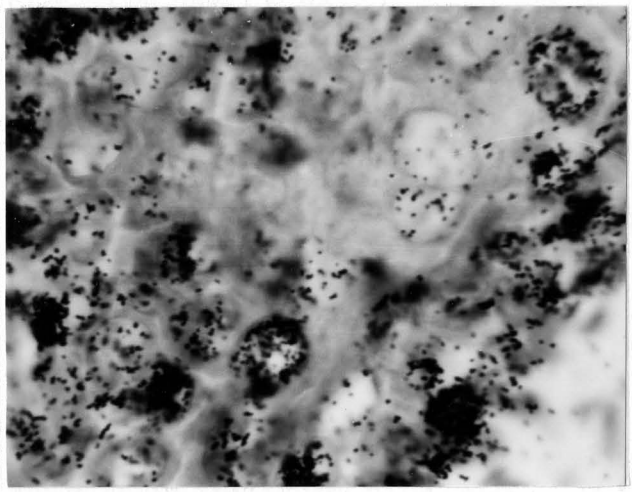
FIGURE 2 Some representative examples demonstrating the extent of radiolabeling found in the donor animals. x1000 H & E



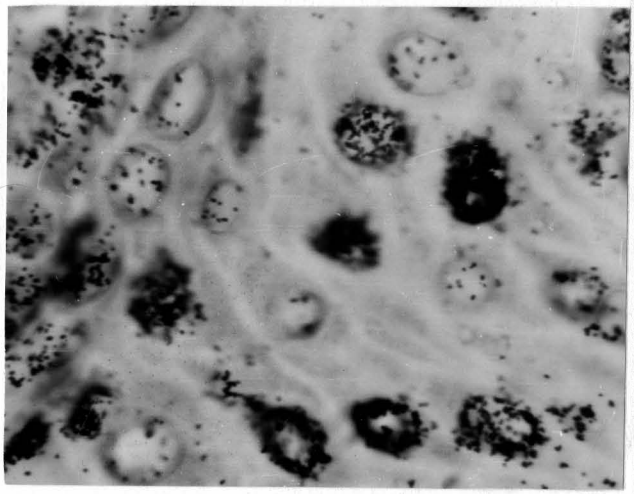
C. Cervical Lymph Node



D. Bone Marrow



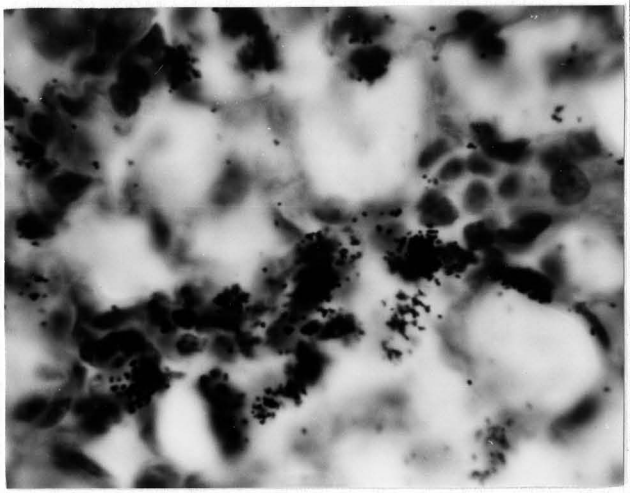
E. Oral Mucosa



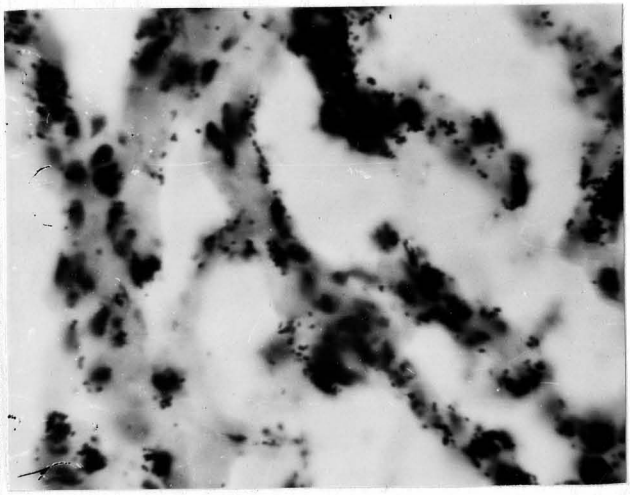
F. Tongue

FIGURE 2 (continued) Some representative examples demonstrating the extent of radiolabeling found in the donor animals. x1000 H & E

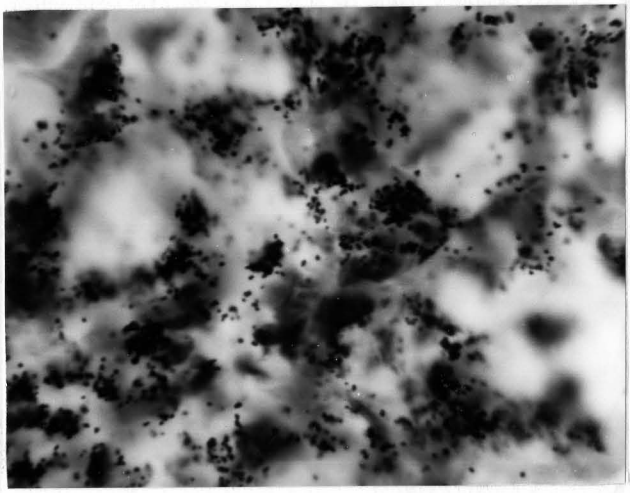
G. Small Intestine



H. Submandibular Salivary Gland



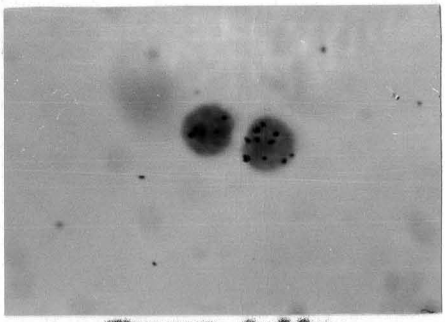
I. Lung



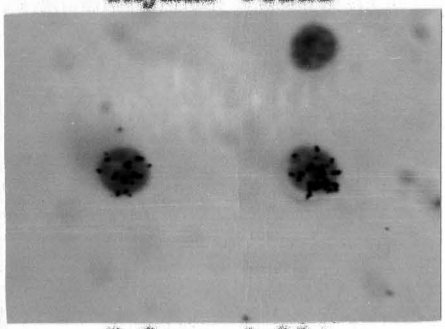
J. Kidney

FIGURE 2 (continued) Some representative examples demonstrating the extent of radiolabeling found in the donor animals. x1000 H & E

Representative examples demonstrating the extent of radiolabeling among the thymus and spleen cell suspensions which were prepared from donor animals, and injected into recipient animals, are shown in figure 3. The intensity of labeling generally appeared somewhat less among thymocytes as compared to spleen cells.



Thymus Cells



Spleen Cells

FIGURE 3 Representative examples of radiolabeling of the spleen and thymus cell suspensions prepared from donor animals receiving 1μc of tritiated thymidine per gram body weight, daily for eight days. x1000 H & E

TABLE 1 The relative radioactivity and percentage of radiolabeled cells of the thymus and spleen cell suspensions prepared from donor animals receiving 1μc of tritiated thymidine per gram body weight daily for eight days.

Cell Suspensions	dpm* 2 mg. dry weight sample	Percentage of radiolabeled cells
Thymus	3710	32
Spleen	3886	53

* dpm: disintegrations per minute

The following is a list of tissues observed in recipient animals which had received injection of radiolabeled thymus and spleen cell suspensions. The cell suspensions had been prepared from donor animals which had received 1μc of tritiated thymidine per gram body weight daily for eight days. The recipient animals were sacrificed seventy-two hours after receiving radiolabeled cell suspensions.

Spleen and intestinal lymph nodes: Frequent heavily labeled cells were observed in the spleen and to a lesser extent in intestinal lymph nodes of the recipient animal receiving radiolabeled spleen cells (figure 4 C, D). In contrast, the spleen and intestinal lymph nodes of the recipient animal receiving radio-

labeled thymus cells demonstrated both fewer labeled cells and a lower number of grains per cell (figure 4 A, B). The majority of labeled cells in the spleen and intestinal lymph nodes were observed perifollicularly. The intensity of the radiolabel appeared to be in a range compatible with labeling observed in the injected radiolabeled cell suspensions (figure 3). No morphological differentiation between donor and recipient cells could be made.

Thymus and cervical lymph nodes: The thymus and cervical lymph nodes in both recipient animals, receiving thymus or spleen cell suspensions, showed no evidence of either heavily or frequently labeled cells (figure 5). The labeled cells were found to be in the cortex of the thymus and perifollicularly in the cervical lymph nodes. No morphologic differentiation between donor and recipient cells could be made.

Lung: A low frequency of fairly heavily labeled cells was observed in the lungs of both recipient animals receiving radiolabeled thymus and spleen cell suspensions (figure 6). Those cells which appeared labeled were found within the walls of the alveoli. The labeled cells often appeared to be macrophages, though frequently one could not definitely differentiate, on a morphological basis, between macrophages, lymphocytes, or thymocytes.

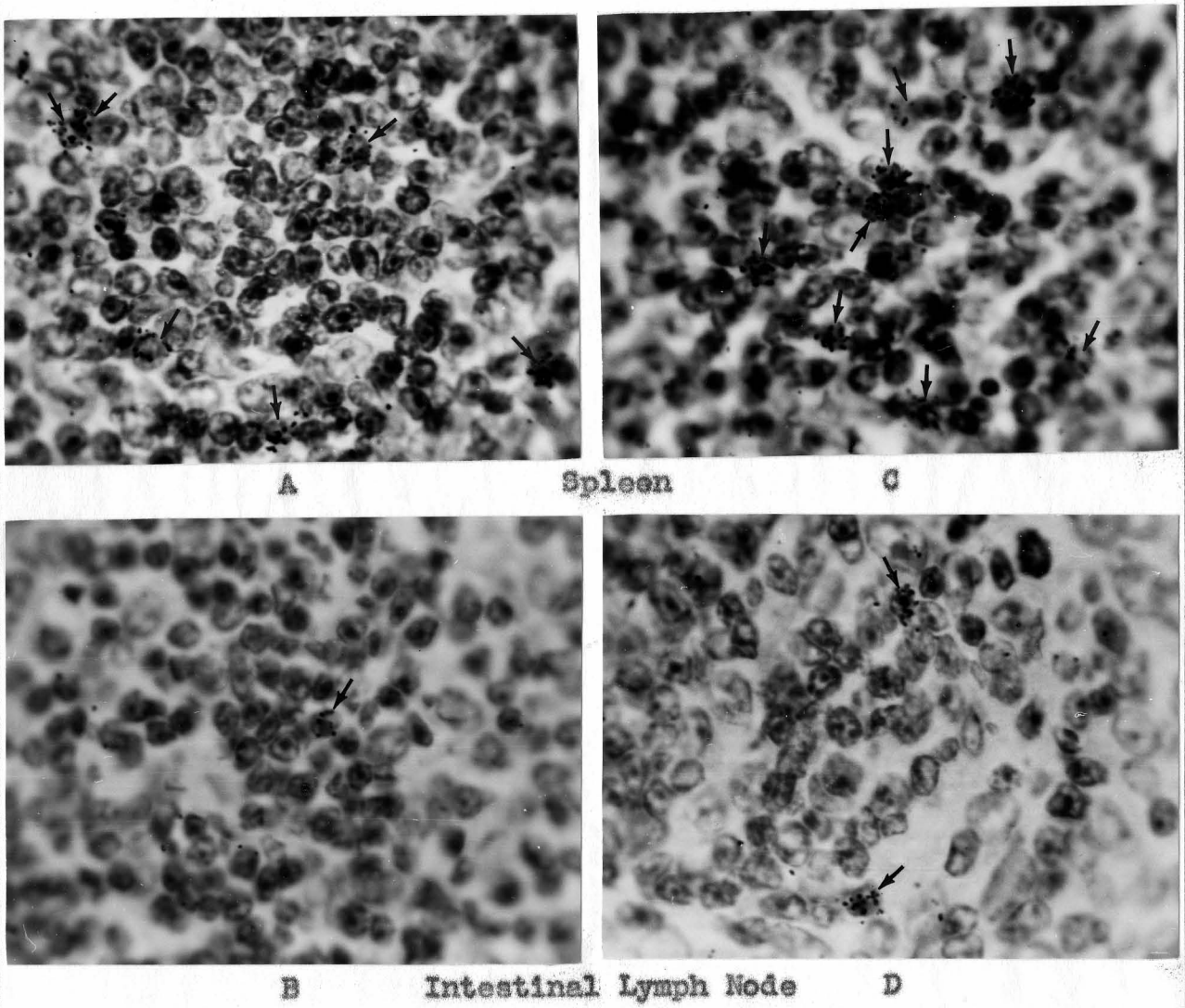


FIGURE 4 Representative examples of the spleen and intestinal lymph nodes of recipient animals receiving thymus (A, B) and spleen (C, D) cell suspensions. x1000 H & E

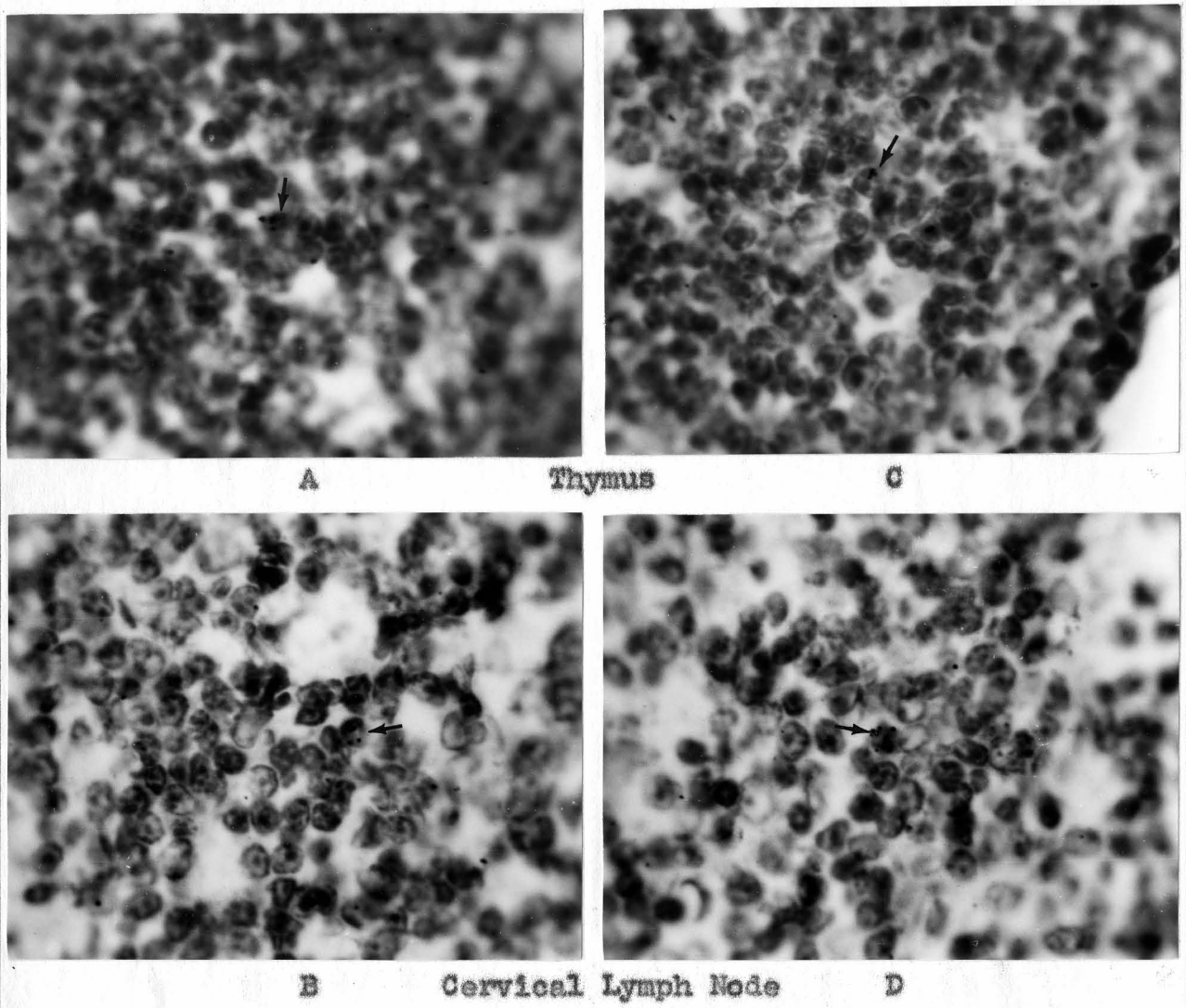
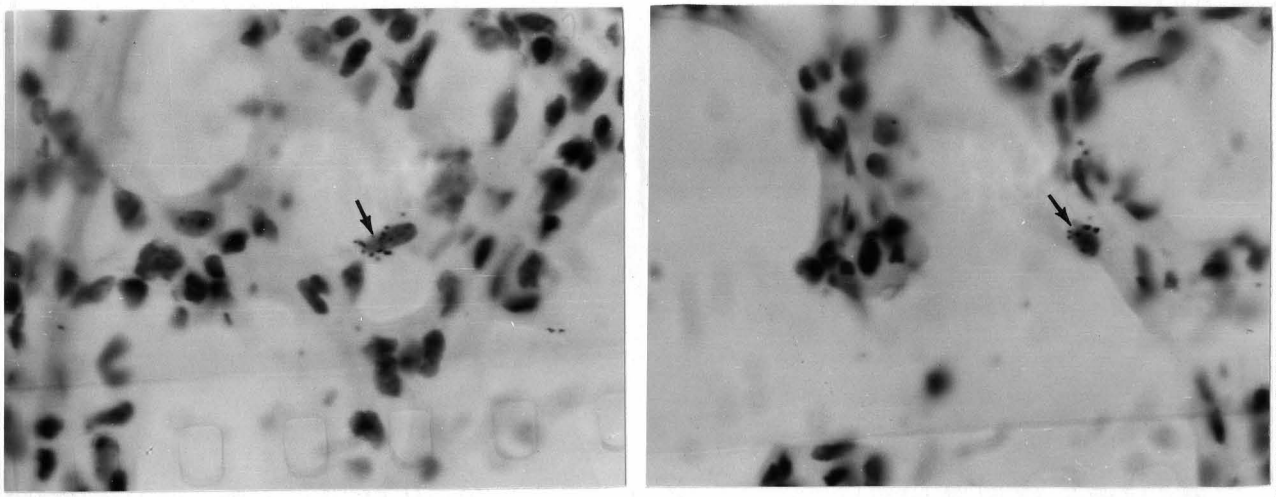


FIGURE 5 Representative examples of the thymus and cervical lymph nodes of recipient animals receiving thymus (A, B) and spleen (C, D) cell suspensions. x1000 H & E



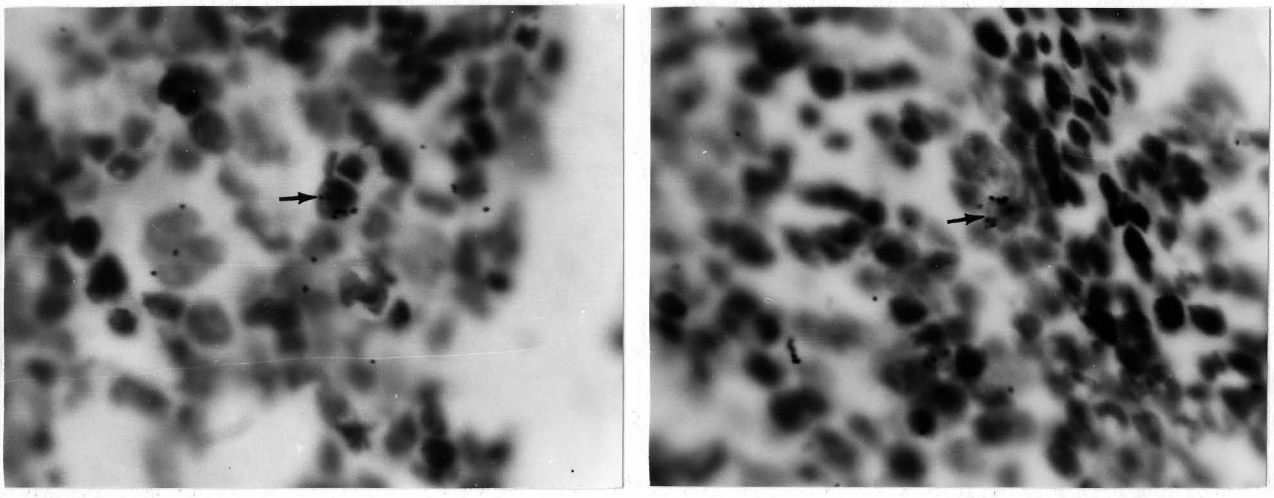
A B

FIGURE 6 Representative examples of labeled cells within the alveoli of lungs of recipient animals receiving radiolabeled thymus (A) and spleen (B) cell suspensions. x1000 H & E

Bone marrow: In both recipient animals receiving labeled thymocytes and spleen cells, the bone marrow demonstrated a few low level labeled hemocytoblasts (figure 7). No other labeled cell types appeared to be present in the sections examined.

Testes: Labeled secondary spermatocytes were found in both recipient animals receiving radiolabeled thymus or spleen cell suspensions (figure 8 A, B). No other cell types were labeled except in the tunica of the testes of the recipient animal receiving radiolabeled spleen cells (figure 8 C, D). Within the tunica of this recipient animal, there were heavily labeled lymphocytes with the same intensity of label as that observed in

the radiolabeled spleen cell suspension (figure 8 D). A few significantly labeled cells, which appeared to be fibroblasts, were also seen in the tunica of the testes (figure 8 C, D).



A B

FIGURE 7 Representative examples of labeled hemocytoblasts in the bone marrow of recipient animals receiving radiolabeled thymus (A) and spleen (B) cell suspensions. x1000 H & E

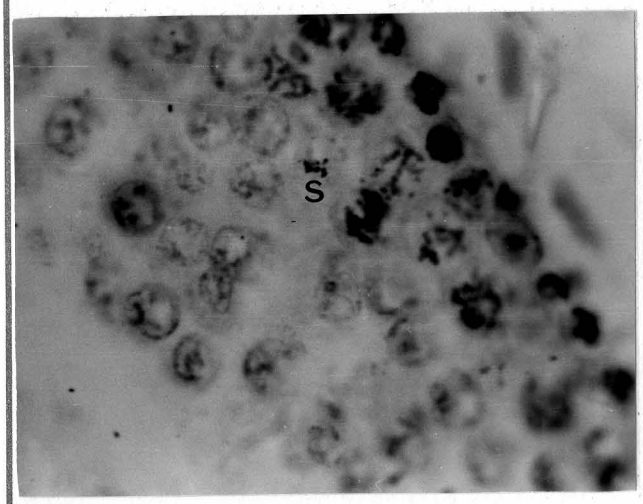
Liver and kidney: Liver cells and kidney tubule cells in both recipient animals were found to be significantly labeled (figure 9). In the sections observed, no cells were labeled, other than liver parenchyma cells and kidney tubule cells

Tongue and oral mucosa: A few significantly labeled cells were observed in the epithelium of the tongue and oral mucosa (figure 10). The labeled cells were in the low prickle cell layer. Similar observations were made in both recipient animals

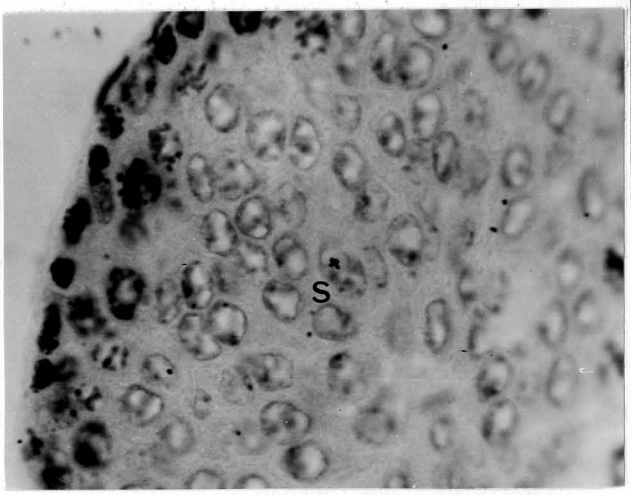
receiving radiolabeled thymus and spleen cell suspensions.

Intestinal mucosa: Occasional cells of the intestinal mucosa were found to contain a low level label (figure 11). The frequency of labeled mucosa cells appeared to be equal in both recipient animals receiving radiolabeled thymus and spleen cell suspensions.

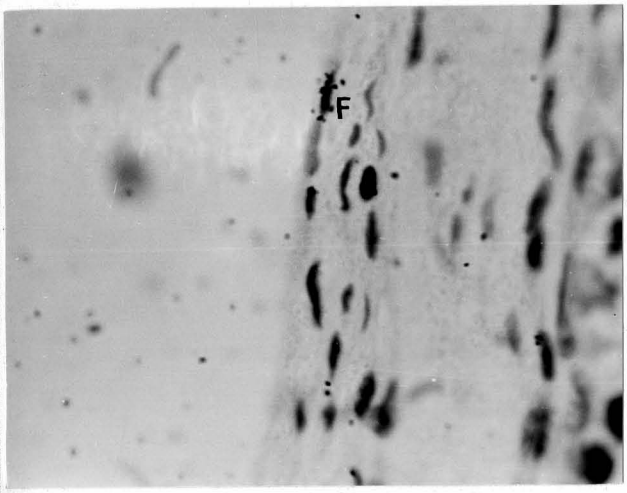
Submandibular salivary gland and blood: Neither of the recipient animals receiving radiolabeled thymus and spleen cell suspensions demonstrated any significant labeling within the submandibular salivary glands or blood. No components of the radiolabeled cell suspensions were observed in either of these tissues.



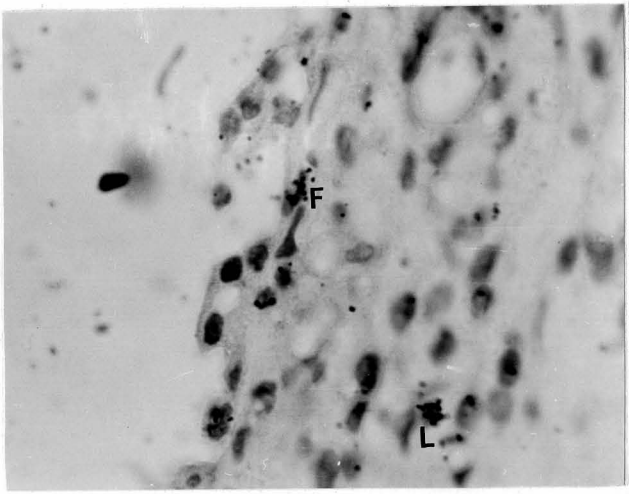
A



B

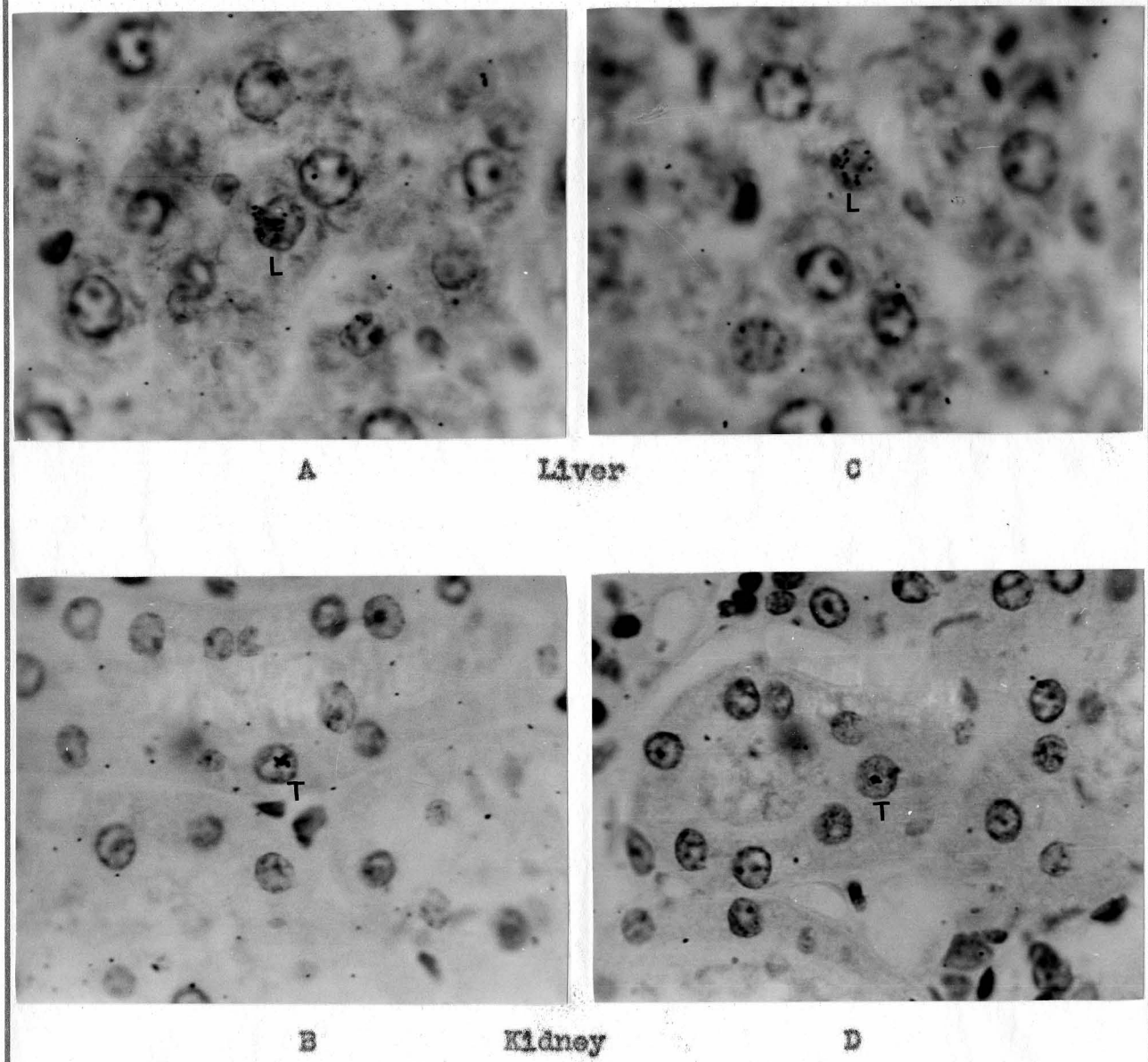


C



D

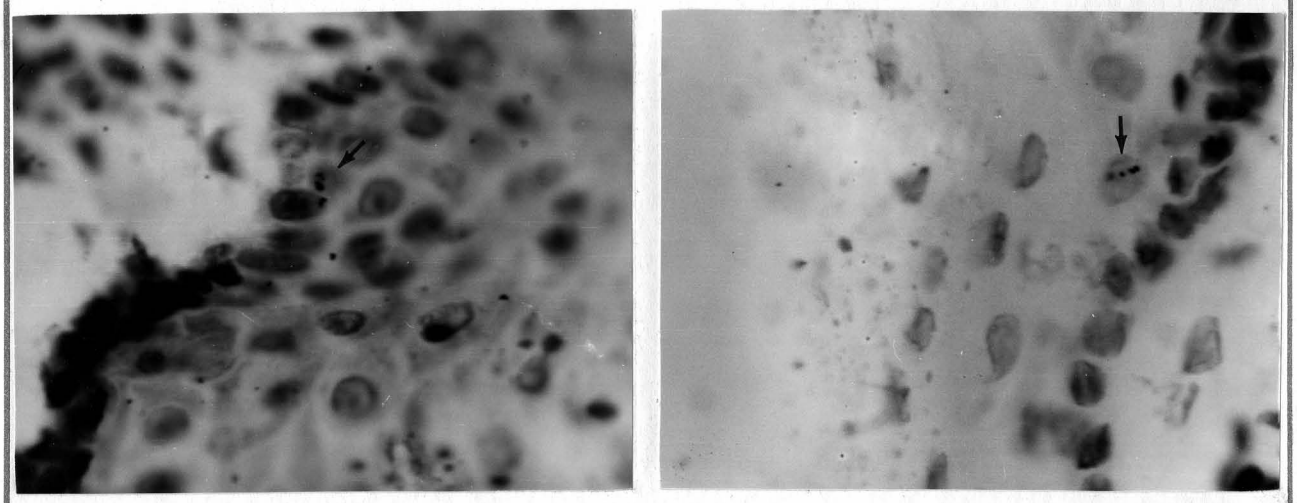
FIGURE 8 Representative examples of labeled secondary spermatocytes (A, B) within the testes and labeled fibroblasts within the tunica of the testes of animals receiving radiolabeled thymus (A) and spleen (B, C, D) cell suspensions. S: secondary spermatocyte, F: fibroblast, and L: labeled lymphocyte. x1000 H & E



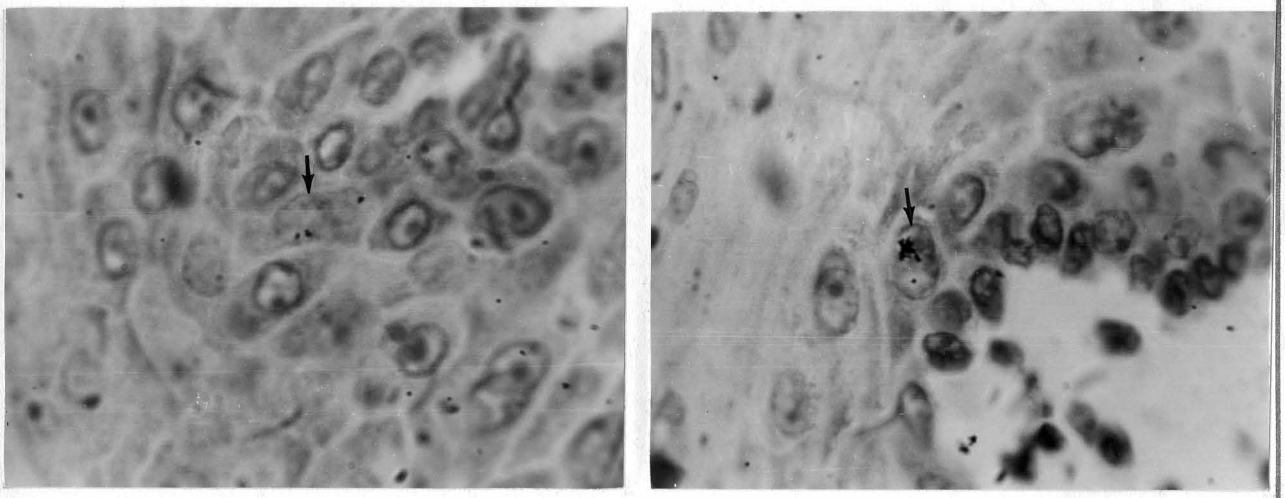
A Liver C

B Kidney D

FIGURE 9 Representative examples of labeled liver cells and kidney tubule cells in recipient animals receiving radiolabeled thymus (A, B) and spleen (C, D) cell suspensions. L: labeled liver cell, T: labeled kidney tubule cell. x1000 H & E

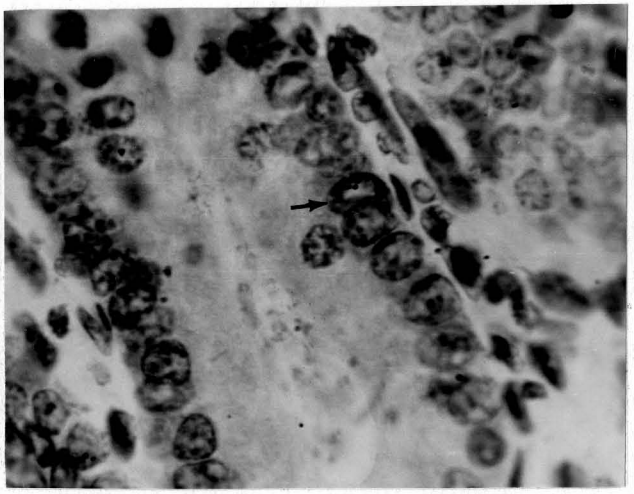


A Tongue C

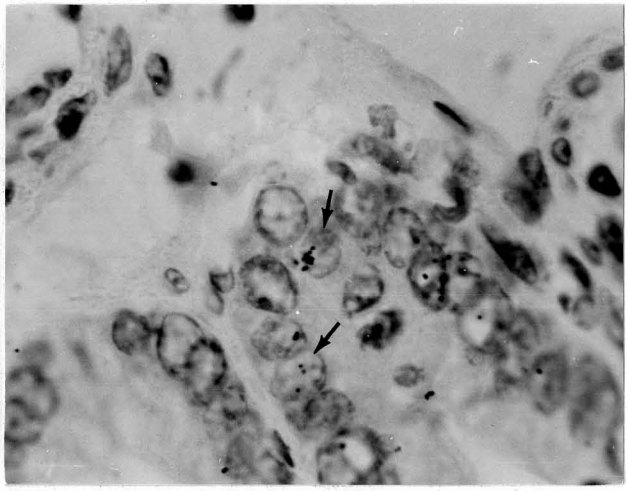


B Oral Mucosa D

FIGURE 10 Representative examples of labeled epithelial cells in the tongue and oral mucosa of recipient animals receiving radiolabeled thymus (A, B) and spleen (C, D) cell suspensions. x1000 H & E



A



B

FIGURE 11 Representative examples of labeled intestinal mucosa cells in recipient animals receiving radiolabeled thymus (A) and spleen (B) cell suspensions. x1000 H & E

FIGURE 2 The relative radioactivity observed in tissues of the donor and recipient animals.

Tissue 2 mg. dry weight samples	Radioactivity (dpm)* in:		
	Donor animals **	Recipient animals*** receiving suspensions of:	
		Thymus cells	Spleen cells
Thymus gland	7450	14	43
Spleen	14968	133	200
Intestinal lymph nodes and adjacent intestinal mucosa	12782	70	197
Cervical lymph node	11974	10	198
Bone marrow	8273	7	12
Lung	6693	16	38
Testes	11673	6	24
Liver	10918	10	17
Kidney	441	6	16
Tongue	978	12	11
Oral mucosa	1077	11	13
Submandibular salivary gland	2549	13	24
Blood	270	59	16

* : dpm: disintegrations per minute

** : donor animals receiving 1 μc of tritiated thymidine per gram of body weight daily for eight days

*** : recipient animals which had received injections of the radiolabeled thymus and spleen cell suspensions indicated in table 1 and sacrificed seventy-two hours after receiving the injections

CHAPTER V

DISCUSSION

The apparent ease with which tritiated thymidine may be incorporated within cells, and its specificity for desoxyribonucleic acid of the nucleus, is demonstrated by the very high intensity of radiolabel observed within the tissues of the donor animals (figure 2) - these observations support the work of Friedkin, et al (1956), Amano, et al (1950), and Cronkite, et al (1958, 1959).

Watson and Crick (1953) have shown that thymine is one of the four bases making up desoxyribonucleic acid. Friedkin, et al (1956) demonstrated the specificity of tritiated thymidine as a desoxyribonucleic acid precursor. If an animal receives an injection of either a thymus or spleen cell suspension which has been radiolabeled by means of tritiated thymidine, and certain other cells of the recipient animal become radiolabeled; the only apparent source of radioactive material for this new radiolabeling must be the labeled desoxyribonucleic acid of the thymus and spleen cell suspensions. Since desoxyribonucleic acid is metabolically stable once formed within the nucleus of a cell (Hughes 1959), the only explanation for new radiolabeling in the recipient animal is: (1) a dedifferentiation (Bloom 1938, Yoffey and Drinker 1939, Farr 1951, Dameshek 1963) of some cells of the radiolabeled

cell suspension or their progeny into multipotential stem cells and then redifferentiation into another cell type, or (2) the reutilization (Ottesen 1954, Hamilton 1954, 1956, Trowell 1957, Hill and Drasil 1960, Bryant 1962, Rieke 1962), by cells undergoing chromosomal replication, of radiolabeled desoxyribonucleic acid released upon cytolysis of labeled cells in the injected radiolabeled cell suspensions, or their progeny. Radioactivity and labeled cells were in fact observed in various tissues of the recipient animal (table 2, figures 4 to 11) by means of a liquid scintillation counter and autoradiograms.

No attempt was made in this study to support or disprove the hypothesis of dedifferentiation of lymphoid cells and their subsequent redifferentiation into another cell type. However, for the sake of completeness, a few comments will be made with regard to this hypothesis. It appears that serious studies dealing with dedifferentiation and redifferentiation have been conducted in the main within the confines of lymphoid and myeloid tissues.

In this study radiolabeled lymphoid and myeloid cells were only observed within the recipient animals in the spleen, intestinal and cervical lymph nodes, thymus, lung, bone marrow, and tunica of the testes (figures 4 to 8). All the labeled cells, in the tissues mentioned above, appeared to be morphologically similar to those cells found within the injected radiolabeled

cell suspensions, and possessed the same range in grain labeling as the radiolabeled cells of the thymus and spleen cell suspensions. On this basis no redifferentiation of cell types could be observed or supported. What one is probably observing in these tissues is either the presence of cells of the injected radiolabeled cell suspensions and possibly their progeny, or the reutilization of radionucleotides by other lymphoid cells undergoing chromosomal replication. Both of these possibilities probably occured, but the data obtained from this study, with regard to the tissues mentioned above, cannot substantiate this assumption.

Labeled thymocytes and lymphocytes were observed in the spleen and intestinal lymph nodes. In comparison, Fichtelius (1958a, b, 1959) observed that P^{32} labeled thymocytes had a predilection for the spleen and liver while labeled lymphocytes may be found in the bone marrow and intestinal lymph nodes in addition to the spleen and liver.

Radiolabeling appeared within some secondary spermatocytes, liver cells, kidney tubule cells, epithelium of the tongue, and oral and intestinal mucosa cells of the recipient animals (figures 8 to 11). Since only radiolabeled thymus and spleen cell suspensions were injected into the recipient animals, the radiolabeling observed within the above mentioned cell types could only be derived from radiolabeled desoxyribonucleic acid found

in the injected labeled cell suspensions. Since tritiated thymidine is a specific precursor of desoxyribonucleic acid, and desoxyribonucleic acid is metabolically stable, nucleic acids must have been released upon the cellular catabolism of the radiolabeled cell suspensions. The released labeled desoxyribonucleic acid may then have been incorporated within those cells, mentioned above, which were undergoing chromosomal replication.

The radiolabeled desoxyribonucleic acid may have been reutilized in a depolymerized or partially polymerized stage. No comparative observations were made in this study on the efficacy of radiolabeling using polymerized or depolymerized labeled desoxyribonucleic acid. However, the appearance of a substantial label over the nuclei of some apparently newly labeled cells of the recipient animal leads one to speculate that either this radiolabel is due to the reutilization of fragments of polymerized radiolabeled desoxyribonucleic acid, or a concentration of depolymerized radiolabeled desoxyribonucleic acid from the local cytolysis of labeled cells near cells undergoing chromosomal replication, or both. Rieke (1962) demonstrated that more cells may be labeled with a heavier label when polymerized tritiated thymidine labeled desoxyribonucleic acid was made available to growing sarcoma cells as compared to depolymerized radiolabeled desoxyribonucleic acid.

Upon examining the gross radioactivity of the thymus and

spleen cell suspensions with a liquid scintillation counter, it was found that the activity of the two cell suspensions was approximately equal (table 1). However, upon examination of autoradiographs of the cell suspensions, thirty-two percent of the thymus cells appeared labeled with a somewhat less intense label than that observed among the spleen cell suspension (figure 2) in which fifty-three percent of the cells were labeled. This disparity in values between the liquid scintillation counter and autoradiograms is perhaps due to one or more of several factors: (1) the intensity of radiolabel among some of the thymocytes may not have been adequate to activate the silver halide grains in the autoradiographic emulsion, (2) in the process of preparing the cell suspensions, some cells may have been crushed, thus releasing visually indistinguishable radiolabeled desoxyribonucleic acid fragments which may have been is suspension or solubilized, (3) a non-representative fraction of the cell suspension may have been taken in the preparation of cell smears (though this seems improbable, since the suspensions were well shaken before samples were withdrawn), or (4) some unknown human or mechanical error may have occured. It is most probable that some cells may have in fact been labeled with a radiolabel that was not adequate to activate silver halide grains of an autoradiographic emulsion.

No significant visual labeling was observed in autoradiograms

in either the submandibular salivary gland or the blood of recipient animals. However, upon examination of the two tissues by means of a liquid scintillation system, a significant amount of radioactivity was observed as compared to other tissues (table 2) which had also exhibited positive autoradiograms. Either many cells of the blood and submandibular salivary gland were labeled with a radiolabel not adequate to activate the silver halide grains of an autoradiographic emulsion, ar autophosphorescence of the solubilized tissues may have occured, or both. Autophosphorescence of course would activate the fluors of the scintillating solution and cause false positive radioactivity in the liquid scintillation counter.

The results of this study appear to support the hypothesis that desoxyribonucleic acid made available by catabolism of cells may be reutilized by other cells undergoing chromosomal replication. The somewhat dogmatic statements by Potter (1959), Dancis and Balis (1959), and Cronkite, et al (1959), which is accepted in some of the current literature as to the non-reutilization of desoxyribonucleic acid, is questionable in light of the results of this study. Though purines and pyrimidines may be degraded upon reaching the liver via the vascular system, they may certainly be readily available for reutilization upon cytolysis, probably in the relative proximity of other cells undergoing chromosomal replication. In which case, the free desoxyribonucleic acid would

not reach the liver.

This hypothesis concerning the reutilization of desoxyribonucleic acid is supported by the observations, mentioned earlier, of Ottesen (1954), Hamilton (1954, 1956), Trowell (1957), Medawar (1957), DeBruyn (1948), Hill (1959), Kelsall and Crabb (1959), Hill and Drasil (1960), Schorley and Berman (1960), Bryant (1962), and Rieke (1962).

CHAPTER VI

SUMMARY AND CONCLUSIONS

Cell suspensions of thymocytes and spleen cells were prepared from donor animals receiving 1μc of tritiated thymidine (sp. act. 1.9c/mM) per gram body weight, daily for eight days. The cell suspensions were injected intraperitonealy into recipient animals which were sacrificed seventy-two hours later.

Radioactivity and radiolabeled cells were observed in various tissues of the recipient animals by means of a liquid scintillation counter and autoradiographs. Radiolabeling was observed, in the recipient animals, over cell types which were not found in the radiolabeled injected cell suspensions prepared from the donor animals. It was concluded that the source of this radiolabeling in the recipient animals, over cells other than those injected, was derived from radiolabeled thymocytes and lymphocytes of the injected donor cell suspensions, which upon cytolysis released their desoxyribonucleic acid which was reutilized by other cells undergoing chromosomal replication.

Although reutilization of nucleic acid has been definitely demonstrated in this study, the magnitude of this source has not been determined. Therefore, extensive quantitative observations are clearly indicated.

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APPROVAL SHEET

The thesis submitted by Dr. Kenneth R. Goljan has been read and approved by three members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 18 1964
Date

Patrick D Tetz
Signature of Advisor