A STUDY OF TRITIATED THYMIDINE INCORPORATION IN MOUSE FEMUR BONE MARROW DEOXYRIBONUCLEIC ACID

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This is to certify that the

thesis entitled

A STUDY OF TRITIATED THYMIDINE INCORPORATION IN MOUSE FEMUR BONE MARROW DEOXYRIBONUCLEIC ACID

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Lester F Wolterink
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ABSTRACT

A STUDY OF TRITIATED THYMIDINE INCORPORATION IN MOUSE FEMUR BONE MARROW DEOXYRIBONUCLEIC ACID[1]

by Leo Herman Blackwell, Jr.

The incorporation of H^3-thymidine in mouse femur bone marrow deoxyribonucleic acid (DNA) and whole mouse femur was studied by:

1) Extracting DNA from femur bone marrow cells and assaying DNA for tritium, utilizing liquid scintillation counting, and
2) Combusting whole dried femurs to tritiated water and carbon dioxide and assaying the tritiated water by liquid scintillation counting.

The data permit the following observations and conclusions:

1) Tritiated thymidine is actively incorporated into bone marrow DNA both directly from the plasma at a very rapid rate and indirectly from a precursor pool at a much slower rate.
2) The greatest contribution to total DNA turnover in the normal mouse femur marrow is made by the turnover of erythroid cell DNA.
3) A very large endogenous pool of DNA precursors, transported through the plasma to the marrow, must be postulated.

Leo Herman Blackwell, Jr.

4) Additional DNA, as DNA, enters the normal mouse femur from some other source. Tritium label from this source appears in the mouse femur between eighteen and twenty-four hours after injection of tritiated thymidine.

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[1] This work was performed under the auspices of the United States Atomic Energy Commission.

A STUDY OF TRITIATED THYMIDINE INCORPORATION IN MOUSE FEMUR BONE MARROW DEOXYRIBONUCLEIC ACID

By

Leo Herman Blackwell, Jr.

A THESIS

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To my wife, June,

who gave many hours of encouragement and criticism.

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INTRODUCTION AND REVIEW OF LITERATURE

Tritium and Tritiated Thymidine

In 1919, Lord Rutherford (see Rutherford, Chadwich and Ellis, 1951) found the (d,d) reaction produced tritium in addition to helium-3. It was not until 1939 that Alvarez and Cornog proved that helium-3 was stable and tritium was the radioactive component. Tritium can now be produced by pile reactions such as ${}_{3}Li^{6} + {}_{0}n^{1} \longrightarrow {}_{2}He^{4} + {}_{1}H^{3}$. Because tritium is a very weak beta emitter ($E_{maximum}$ = 18 kiloelectronvolts, half life = 12.4 years), it has been utilized for autoradiographic analysis of biological specimens. In 1951, Robertson designed a proportional counting system for tritium analysis. Since that time, liquid scintillation counting systems have been developed. These have made possible the analysis of tritium in biological samples which complement work done utilizing autoradiography.

Thymidine was tritiated by Verly in 1957. The method used was a catalytic exchange between tritium water and thymidine. Friedkin (1960) showed that the preparation produced by Verly, as well as commercial preparations of tritiated thymidine (H^{3}-thymidine) contained tritium in the methyl group. The tritium thus bound is highly stable, and only metabolic reactions are capable of liberating tritium from labeled thymidine introduced into cells (Verly _et_ _al_., 1958).

Specificity of Thymidine for Deoxyribonucleic Acid

Thymidine labeled with Nitrogen-15 is a specific precursor of deoxyribonucleic acid (DNA) in rats (Reichard and Estborn, 1951). Later work by Friedkin, Tilson and Roberts (1956) using thymidine-2-carbon-14 proved that thymidine was incorporated into DNA of bone marrow and other tissues, with only a negligible amount of the radioactivity being incorporated into RNA or components other than DNA. Because of this high degree of specificity for DNA, thymidine has come to be widely used for the study of cell proliferation, since DNA can be tagged only during DNA synthesis (i.e., DNA replication) immediately prior to mitosis.

It has been established that free bases are not utilized in the production of nucleic acids, but that ribosides and ribotides, as well as simple precursors are utilized in nucleic acid formation (Schulman, 1961). Thymidine is degraded to carbon dioxide and β-aminoisobutyric acid (probably in the liver in mammals) (Rubini et al., 1960). The normal pathway for thymidine incorporation prior to the final assembling of new DNA in a proliferating cell system is thought to be by methylating deoxyuridylic acid to thymidylic acid which is then incorporated directly into new DNA (Rubini, Keller and McCall, 1964). When H^3-thymidine is injected into an animal, it is evidently phosphorylated to thymidylic acid for subsequent incorporation into DNA.

Cell Renewal Systems

The study of tissue proliferation began, perhaps, with Hamm and Leeuwenhoek's discovery and description of spermatozoa in 1677 (see Needham, 1963). These authors, however, did not realize that the small cells they observed were the consequence of cell proliferation. Since these early studies, the growth of cell populations in the adult organism has been studied in relation to hormone response, repair after trauma (regeneration in lower animals, repair of radiation damage), and tumor growth. New cells (thus new DNA) can arise only by mitosis, and since some tissues exhibit a greater rate of mitosis than others, DNA replication varies in a similar fashion. Patt (1954), and other workers in the field of radiation biology, noted that some tissues (gut, blood cells, skin) are constantly renewed, and that there exists a steady state between production and loss, as long as the system is not perturbed. Failure of these cell systems to produce new cells after irradiation is due to the blockage of mitosis, as well as death of proliferative cells.

In 1956, Leblond and Walker applied the term "cell renewal system" to these cell populations that are kept in normal balance by continuous and equal rates of production and loss. These renewal systems may be divided into further compartments as follows:

Birth ⟶ Proliferation ⟶ Differentiation ⟶ Function ⟶ Death and Removal.

The birth compartment is represented by an undifferentiated cell (called a stem cell) which can undergo mitosis (thus producing new cells and therefore new DNA) and give rise to a somewhat more differentiated cell capable of further mitosis. The proliferative compartment is represented by the cells differentiated from the stem cells. The function of the cells in the proliferative compartment is to amplify the number of cells which will eventually become the mature functional components of the cell system. Thus, the major characteristic of the proliferative compartment is mitosis, although some differentiation also takes place. The proliferative compartment, and the birth compartment, are the only sourcesof new DNA. Obviously, the chief site of DNA replication is the proliferative compartment which is numerically much larger than the birth compartment. Although there may be several different recognizable cells in the proliferating compartment, they all undergo similar cell cycles.

$$\begin{tikzcd}
G_1 \arrow[r] & S \arrow[d] \\
M \arrow[u] & G_2 \arrow[l]
\end{tikzcd}$$

In most mammalian cell renewal systems, the mitotic time (M) is about 1 hour, the time (G_2) between DNA synthesis (S) and mitosis is about 1 to 2 hours, and the DNA synthesis time (S) is about 4 to 10 hours. These times are thought to be fairly constant with G_1 being the variable between cell cycle lengths in different cell systems (Patt and Quastler, 1963).

Cells in the differentiating and functional compartments are not capable of further DNA synthesis, therefore, to keep the system in balance, new cells (or new DNA) must be furnished from the proliferating compartment, to replace cells lost from the functional compartment.

In general, all cells in a particular renewal system contain about the same amount of DNA per nucleus. Consequently, a chemical DNA analysis of a tissue includes both the DNA capable of replication (i.e., the proliferative compartment) and the DNA incapable of replication (i.e., the differentiating and functional compartments).

Until radioactive isotopes became readily available, the only way to study the dynamics of a cell renewal system was to evaluate the mitotic index $(\frac{\text{number of cells in mitosis}}{\text{total number of cells in the population}})$ for recognizable cell types. With an assumed mitotic time (usually 1 hour) and the mitotic index, one can calculate the turnover time (the time taken for the replacement of a number of cells equal to the whole population). If the number of cells produced per unit of time is known, the total number of cells in the population can be estimated (Patt, 1957).

$N = \frac{Pt}{M}$ where: N = Number of cells in population

t = Mitotic time

P = Cells produced per unit time

M = Mitotic index

The difficulties one encounters by analyzing a cell renewal system in this fashion are associated with such things as the assumed mitotic time and the growth fraction of the population (i.e., that fraction of a cell population which will undergo DNA replication to the total population). If, for example, certain of the cells that appear to be capable of dividing never participate in cell division, the mitotic index will be underestimated (Mendelsohn, 1960). In effect, two populations of morphologically like cells may exist, those that will divide and those that will not divide.

Labeling cells with radioactive markers has enhanced the study of cell renewal systems since by utilizing autoradiography one can actually follow a population of given cell types as it moves from one compartment to another. In addition, one can obtain the labeling index $\left(\frac{\text{total number of cells labeled}}{\text{total number of cells in population}}\right)$. The duration of DNA synthesis can be estimated rather precisely using timed autoradiography (Maloney, Patt and Weber, 1962; Wimber, 1963). Knowing the labeling index and the DNA synthesis time, one can again calculate the turnover time. However, the problem of the growth fraction is still present. These parameters do provide a precise estimation of the proliferation rate of the population.

$$\text{Proliferation rate} = \frac{\text{Number of cells labeled}}{\text{DNA synthesis period}} \quad \text{(cells/hour)}$$

If the relative number of cells of different types in a mixed population is known, their relative proliferation rates can be determined (Patt and Maloney, 1963).

Modern studies of cell renewal systems are usually based on one or both of the above methods of analysis. Some studies have followed the activity of a radioactive precursor in DNA synthesis by standard counting methods, but until tritiated thymidine became available, there were no tracers which were specific for incorporation into DNA alone. Therefore, the interpretation of those results was difficult.

Because of the high degree of specificity of thymidine for DNA as discussed above, many workers have used tritiated thymidine to study cell proliferation (Hughes et al., 1958; Bond et al., 1959; Patt and Maloney, 1959). Generally, they have made the following assumptions (Cronkite et al., 1959a):

"1) The tritium label on thymidine does not exchange.

2) Thymidine base does not exchange after incorporation into DNA.

3) DNA turnover is solely the result of mitosis and cell death.

4) Re-utilization of tritium labeled materials in DNA synthesis is insignificant.*

5) Re-utilization of large chunks of DNA is unlikely in most cell renewal systems.

6) DNA synthesis in normal cells destines a cell to divide once again.

7) Tritium-labeled thymidine is uniformly distributed throughout the body and is either incorporated into DNA or degraded.

8) The effective availability time of H^3-thymidine for DNA synthesis is short and a small fraction of the time for synthesis of DNA.

9) There is no significant radiation injury of these cells.

10) In vitro labeling determines the proliferative potential of normal cells.

11) In vivo labeling after a single intravenous injection makes possible the study of the kinetics of cell proliferation."

The validity of several of these assumptions has been questioned. Some of these (4, 5 and 8) will be considered in this study.

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*In Cronkite's list of assumptions, this reads "significant." This is evidently a misprint. See some of his other papers (Cronkite et al., 1959b; Cronkite et al., 1960).

Studies of Deoxyribonucleic Acid Synthesis with Tritiated Thymidine

In Vitro Studies

The fate of H^3-thymidine *in vitro* has been studied utilizing tissue cultures and autoradiography of bone marrow cells of various animals (Rubini *et al*., 1962). It was found that if cold thymidine was added at the same time as tritiated thymidine almost complete suppression of labeling occurred. If, on the other hand, cold thymidine was added 20 minutes after H^3-thymidine, maximum labeling was not affected. The implication is that only 20 minutes are required to incorporate thymidine into DNA. In other words, "flash labeling" is characteristic of such systems.

After 1 hour of incubation, the majority of the activity was present still as H^3-thymidine (as determined by chromatography), but when fresh cells were added to the medium, no labeling occurred. If fresh H^3-thymidine was added, additional labeling did occur, Rubini *et al*. (1962) postulated that in the "used" fraction, (i.e., the "thymidine" was still present but unavailable) there was a build-up of "cold" deoxyuridine and thymidine which diluted the specific activity of the H^3-thymidine, so that further labeling did not occur, despite constant incorporation of thymidine into DNA. These authors calculated that there is an endogenous pool of thymidine which contained two times the amount of thymidine necessary for DNA synthesis during the hour of the culture. Cronkite *et al*. (1959b) calculated that *in vivo* not all cells could have an endogenous pool of thymidine in view of the grain counts one observes over a cell on an autoradiograph. The results of the experiments reported in this work question the validity of this calculation, at least for cells of the bone marrow.

In Vivo Studies

Studies in humans on the blood clearance of H^3-thymidine (and the clearance of non-volatile breakdown products of H^3-thymidine) have utilized liquid scintillation counting (Rubini *et al*., 1960). The subsequent appearance of tritium-labeled DNA in bone marrow was observed autoradiographically. They showed that the blood clearance of non-volatile tritium activity was very rapid, with 90 percent being lost in the first minute. They could not account for 50 percent of the material injected, and assumed that it was incorporated into DNA. Rubini *et al*. (1960) describe half-times for loss of activity from the plasma for as long as 30 minutes. The assumption is that some semi-logarithmic function with time should characterize plasma disappearance kinetics. However, it appears that their data are better represented by a power function, as will be described later.

The possible existence of an endogenous thymidine pool or substrate pool which could be labeled by the initial injection of H^3-thymidine and thus feed label into replicating DNA over an extended period has been ignored by most investigators. There is some evidence in the literature which indicates that some DNA precursor pool may be labeled with a single injection of H^3-thymidine and then feed into DNA for an extended period. The data from erythroid element proliferation in bone marrow is a case in point.

Hypertransfused mice have been shown to have red cell production completely suppressed (Filmanowicz and Gurney, 1961), and the bone marrow is almost entirely free of any erythroid cells (Schooley and Garcia, 1962). When erythropoietin is given to such animals, a wave of erythropoiesis follows, as seen both from the appearance of nucleated erythroid cells in the marrow and from the uptake of radioactive iron in hemoglobin. When Schooley and Garcia (1962) and Gurney (1962) injected H^3-thymidine into hypertransfused mice, and 6 hours later gave erythropoietin, they found labeled erythroid precursor cells in the first wave of erythropoiesis initiated. Since the stem cell would not have been stimulated to divide until 6 hours after the injection of H^3-thymidine, either the stem cell must have been in active proliferation, or there was a labeled substrate pool capable of feeding labeled precursor into DNA.

Cronkite *et al*. (1959b) and Quastler (see Lajtha, 1963) present flow diagrams that include the possibility of such a pre-DNA pool. Figure 12 (in the Discussion section) depicts a possible flow of H^3-thymidine in the bone marrow. Presumably, this pattern would be similar for all cell renewal systems (e.g., gut, skin, gonads).

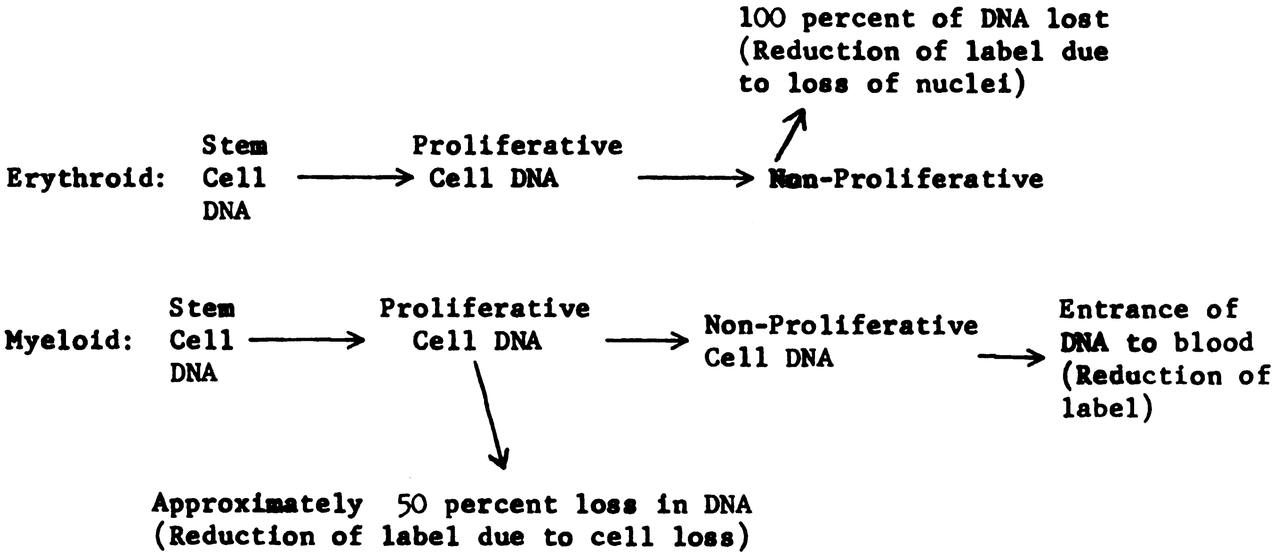
A thorough evaluation of the time during which H^3-thymidine and its breakdown products are available for incorporation during DNA synthesis cannot be found in the literature. This thesis will specifically evaluate these aspects for the case of mouse femur bone marrow cells. In the bone marrow, there are at least two cell renewal systems (myeloid and erythroid). Each is in a steady state, normally. Consequently, total DNA will remain constant in the cases under discussion. Tritium label, however, will change with time, thus providing a true tracer analysis of the over-all DNA kinetics.

General Scheme for Bone Marrow Cell Deoxyribonucleic Acid Turnover

Any H^3-thymidine activity incorporated into erythroid DNA should be lost when the maturing red blood cells lose their nuclei. The time for the loss of the red cell nuclei could be as short as 10 hours or as long as 20 hours after injection of labeled thymidine (Bond *et al*., 1962). However, labeled orthochromatic normoblasts are seen as early as 3 hours (Bond *et al*., 1959). Therefore, if the nucleus is lost over the "life" of the orthochromatic normoblast population rather than abruptly (just before this labeled portion of the population becomes reticulocytes), the loss of labeled DNA could begin 3 hours after injection. Results of chemical determination of DNA activity after a single injection of H^3-thymidine show that the activity incorporated into rat bone marrow DNA (i.e., DNA specific activity) does not change until granulocytes are lost to the blood at 48 hours (Bond *et al*., 1962). Work by Steel (1962) confirms this observation. This implies either reutilization or some other feed-in of label to the bone marrow DNA since no loss in DNA specific activity is observed when red cell nuclei would have been lost. The fact that the activity does not drop in bone marrow DNA in rats is attributed to reutilization of label by Feinendegen *et al*. (1964). This is based on the assumption that 50 percent of the thymidine is retained in the animal as tritiated DNA.

In addition to the loss of bone marrow activity that can be predicted from red cell production, Patt and Maloney (1963), in a model for granulocyte production, show that there could be a substantial loss due to "ineffective" production. Autoradiographic analysis of bone marrow from dogs given tracer amounts of H^3-thymidine indicates that about twice as many cells are produced by the proliferating granulocytes as appear in the later non-mitotic stages. Therefore, loss in DNA activity should occur due to loss of granulocyte precursor DNA as well as erythroid precursor DNA before DNA is lost to the blood when mature granulocytes leave the marrow.

A simplified model of bone marrow cell production in terms of loss of DNA label after injection of H^3-thymidine would be as follows:



Objective of Thesis

From this review of the literature, it is evident that workers using H^3-thymidine in vivo have considered it to be a "flash label" (i.e., a labeling period of about 1 hour) for tagging DNA in the study of proliferating cell systems. However, in vitro studies using bone marrow cells show that there is an endogenous pool large enough to supply two times the thymidine necessary to replicate the DNA in these cultures (Rubini et al., 1962). The degree of erythroid labeling obtained in hypertransfused animals 6 hours after H^3-thymidine injection (Schooley and Garcia, 1962; Gurney, 1962) can better be explained by a continuous feed-in of labeled precursor.

In this work a comparison is made between the tritium activity incorporated into femur bone marrow cell DNA versus the tritium activity incorporated into the total femur following a single injection of H^3-thymidine. The analysis of the whole femur should yield information regarding tritium activity in excess of that incorporated into femur bone marrow cell DNA, since total non-volatile H^3-thymidine activity can be analyzed. The far greater part of DNA of the femur is in the bone marrow; therefore, an analysis of femur activity should represent the activity in the femur bone marrow DNA. If these activities are not the same, then perhaps they can be resolved in the light of the above models and a labeled substrate pool feeding into DNA. In addition, studies have been carried out in hypertransfused animals to obtain some information regarding the difference between erythrocyte and granulocyte production in mouse bone marrow, to assess the DNA turnover of these components.

MATERIALS AND METHODS

Animals

Female Carworth Farms CF-1 mice, or Argonne stock (ANL/CF-1) mice derived from Carworth Farms CF-1 were used throughout. The animals were housed in air-conditioned quarters at 73 ± 2 degrees Fahrenheit, 45 ± 5 percent humidity and kept on a 12-hour light -- 12-hour dark schedule (6 AM - 6 PM). A standard commercial diet and water were available *ad libitum*.

Mice from outside the Laboratory were isolated for 10 days to be sure they were free from disease. Animals from the Argonne stock were checked routinely from the time of birth for any pathogens. Animals selected for weight and age (21 ± 1.5 grams throughout the experiment; 6-8 weeks old) were caged 3 to a cage and left for several days before a particular experiment was begun.

Sacrifice

Animals were anesthetized with 0.25 milliliter of Nembutal (6 milligrams/milliliter) intraperitoneally and pinned to a cork board. An incision was made on the left side, beginning at the costal margin and continuing to the axilla. The brachial artery was then severed and the blood allowed to flow into the pocket formed by the reflected skin. The blood was collected in a heparinized tube for further analysis or discarded. After bleeding stopped, both femurs were removed and processed by one of the procedures indicated below.

Blood

A sample of blood was taken for a hematocrit, then smears were made on all blood samples. These slides were fixed for five minutes in 100 percent methyl alcohol, then stained with Wright's stain for differential counts or saved for preparing autoradiographs. A sample of blood was taken and a leukocyte count done on an electronic cell counter. A Model A Coulter Electronic Cell Counter was used for both leukocyte counts and nucleated marrow cell counts. Usually a 1:500 dilution was used, although if there was an extremely low or high count, appropriate dilutions were made. A 0.5 percent solution of saponin was used to lyse erythrocytes. The procedure and accuracy in the use of the Coulter counter are discussed in several reviews (Coulter, 1956; Brecher, Schneiderman and Williams, 1956; Mattern, Brackett and Olsen, 1957; Richar and Breakell, 1959).

In an early experiment, blood was taken at various times after an injection of H^3-thymidine and centrifuged to separate the plasma from the cells. A 0.1 milliliter sample of plasma was removed and the radioactivity determined as outlined below in the discussion of radioactivity counting.

DNA Extraction

The ends were snipped from the femur and the marrow expelled by positive pressure applied by forcing 0.5 milliliter of physiological saline through a 25 gauge needle. The marrow plug was flushed back and forth through the syringe several times to

break it up as much as possible. The cells were then washed twice with physiological saline and counted on the Coulter counter. The calibration and reliability of the Coulter counter for the bone marrow cells as counted in these experiments are given in Appendix I.

After the aliquot of cells was removed from the femur for the total nucleated cell count, the rest of the cells were taken and the DNA extracted (see also Appendix II), using a modification of Schneider's method as follows (Schneider, 1945):

1) Add 1 milliliter cold 10 percent trichloroacetic acid to cells, then centrifuge for 15 minutes at 2000 revolutions per minute (rpm) in a refrigerated centrifuge.
2) Decant supernatant.
3) Add 2 milliliters 5 percent trichloroacetic acid to precipitate (shake well).
4) Heat in sand bath at 90° Centigrade for 30 minutes.
5) Cool and centrifuge at 2000 rpm for 10 minutes.
6) Transfer supernatant (DNA) to a 5 milliliter volumetric flask.
7) Rinse extraction tube and precipitate with 2 milliliters 5 percent trichloroacetic acid and add to volumetric flask.
8) Make flask up to volume with 5 percent trichloroacetic acid.
9) Take 0.5 milliliter for diphenylamine reaction.
10) Take 4 milliliters for counting radioactivity.

Diphenylamine Reaction

The diphenylamine reaction was done following Burton's Method (Burton, 1956) (see also Appendix III). All samples were run with a DNA standard to check the consistency of the reaction from day to day.

1) To 0.5 milliliter sample add 1 milliliter diphenylamine reagent (1.5 grams diphenylamine, 100 milliliters acetic acid, 1.5 milliliters sulfuric acid) (at the time of use add 0.010 milliliter aqueous acetaldehyde - 16 milligrams/milliliter - to each 20 milliliters of reagent).
2) Mix well and incubate at 30° Centigrade for 20 hours.
3) Read on Coleman Junior Spectrophotometer at 600 millimicrons.

Radioactivity Counting

A Packard TriCarb Liquid Scintillation Counter was used to count the radioactivity in each sample (see also Appendix II).

1) The 4 milliliter aliquot was dried overnight in a vacuum dessicator.
2) One milliliter of hyamine-10X (1 molar solution of p-diisobutyl-cresoxyethoxyethyl dimethylbenzylammonium hydroxide in methanol) was added. The reaction was allowed to proceed for six hours.
3) Fifteen milliliters of scintillator were added and mixed well with the sample. The scintillator consisted of 4 grams of 2,5 Diphenyloxazole (PPO) and 50 milligrams of 1,4-bis 2 (4-methyl-5-phenyloxazolyl) benzene (POPOP) in 790 milliliters of toluene and 250 milliliters of ethanol.

4) The samples were cooled overnight in the TriCarb counter, then counted.

5) An internal standard (0.025 milliliter of a standard tritiated toluene solution containing 2.34 x 10^6 disintegrations/minute/milliliter) was used to correct for quenching.

6) The activity in each sample was computed by comparison to a known standard (0.01 milliliter of the standard tritiated toluene solution used above in 15 milliliters of scintillator).

Femur Combustion

Femurs that were removed for combustion were cleaned of as much soft tissue as possible. The bones were then placed in 50 milliliters of physiological saline and left for several hours. The saline was then changed and the bones left in the fresh saline overnight. This was done to remove any non-volatile activity that may have been in the body fluids. The bones were then washed with distilled water and placed in an oven and dried for 2 days at 90° Centigrade to remove all volatile tritium.

Combustion Technique

A technique for the combustion of samples for tritium analysis has been developed from the Schöniger analysis (Schöniger, 1955) (see also Appendix IV). The technique is as follows:

1) The dried bones are wrapped in black paper and placed in a platinum basket (see Figure 1).
2) A heavy walled two liter Erlenmeyer flask is flushed with pure, dry oxygen.
3) The basket containing the sample is hung on the end of the funnel and placed in the flask.
4) A lamp is then used to ignite the black paper.
5) The sample is thus combusted to water and carbon dioxide.
6) The flask is removed to dry ice and left for 1 hour.
7) The flask is then removed to wet ice for 30 minutes.
8) Thirty-six milliliters of scintillator are added through the funnel and mixed well with the sample (tritiated water).
9) Two 15 milliliter aliquots are then taken for counting in the TriCarb counter.

Tritiated Thymidine

Tritiated thymidine was obtained from Schwartz BioResearch, Inc. with a specific activity of 6 curies/millimole, and activity of 1 millicurie/milliliter. Further dilutions were made in sterile saline, so that the final activity was 50 microcuries/milliliter. The mice were injected with 0.5 microcurie per gram of body weight via the tail vein.

FIGURE 1. COMBUSTION FLASK ARRANGED FOR IGNITION. THE INSERT (UPPER LEFT) SHOWS THE PLATINUM BASKET, BLACK PAPER AND SAMPLE BEFORE WRAPPING AND PLACING AT THE END OF THE FUNNEL.

Hypertransfusion

The mice were made polycythemic by the method described by Jacobson, Goldwasser and Gurney (1960). Old CF-1 or ANL/CF-1 male or female mice were sacrificed as described for the experimental animals. The blood collected from these was centrifuged at 3000 rpm for 15 minutes. The plasma and buffy coat were removed. The red cells remaining were washed three times with physiological saline. After the last wash, the cells were adjusted to a hematocrit of 90 percent. The recipient mice were injected intraperitoneally with 0.5 milliliter fresh cells twice a day for two days. On the fourth day after the initial injection, the animals received two more 0.5 milliliter injections. On the sixth day after the initial injection, all the animals had hematocrits of 70 to 80 percent and the experiment was begun.

Autoradiography

Autoradiographs of blood smears were prepared by placing Kodak AR-10 stripping film on the slides and exposing for three weeks. The film was developed with Kodak-D-19 developer and fixed with Kodak Acid Fixer (Pelc, 1956). After washing and drying the cells were stained with a modified Giemsa Stain (Gude, 1955).

RESULTS

Time for Maximum Uptake of Tritiated Thymidine Activity into Femur Deoxyribonucleic Acid

Two experiments were done to test the rapidity of thymidine uptake into femur DNA. The first experiment was similar to an experiment reported by Rubini et al. (1960). Rubini and co-workers measured the plasma clearance of non-volatile tritium activity in humans. When they analyzed their data as a semi-logarithmic function, they obtained a three component curve. The first two components had half-times of 0.2 and 1 minute respectively. The third component had a half-time of 30 minutes. These workers pointed out that the rapid disappearance of plasma activity could not be correlated with any anatomical or biochemical compartment such as degradation in the liver or uptake by proliferating cell systems and that at 1 minute after injection the plasma concentration corresponded to a dilution as large as the total body water.

The plasma clearance data obtained in the present work, as well as Rubini's, are better represented by a power function than by an exponential function. Figure 2 presents a log-log plot of the results of the present experiments. In these experiments, the plasma activity decreases from about 0.15 microcuries/milliliter at 15 minutes to about 0.010 microcuries/milliliter at 4 hours, indicating that loss occurs very rapidly from the plasma, presumably to cells in DNA synthesis and other sinks for thymidine accumulation or breakdown.

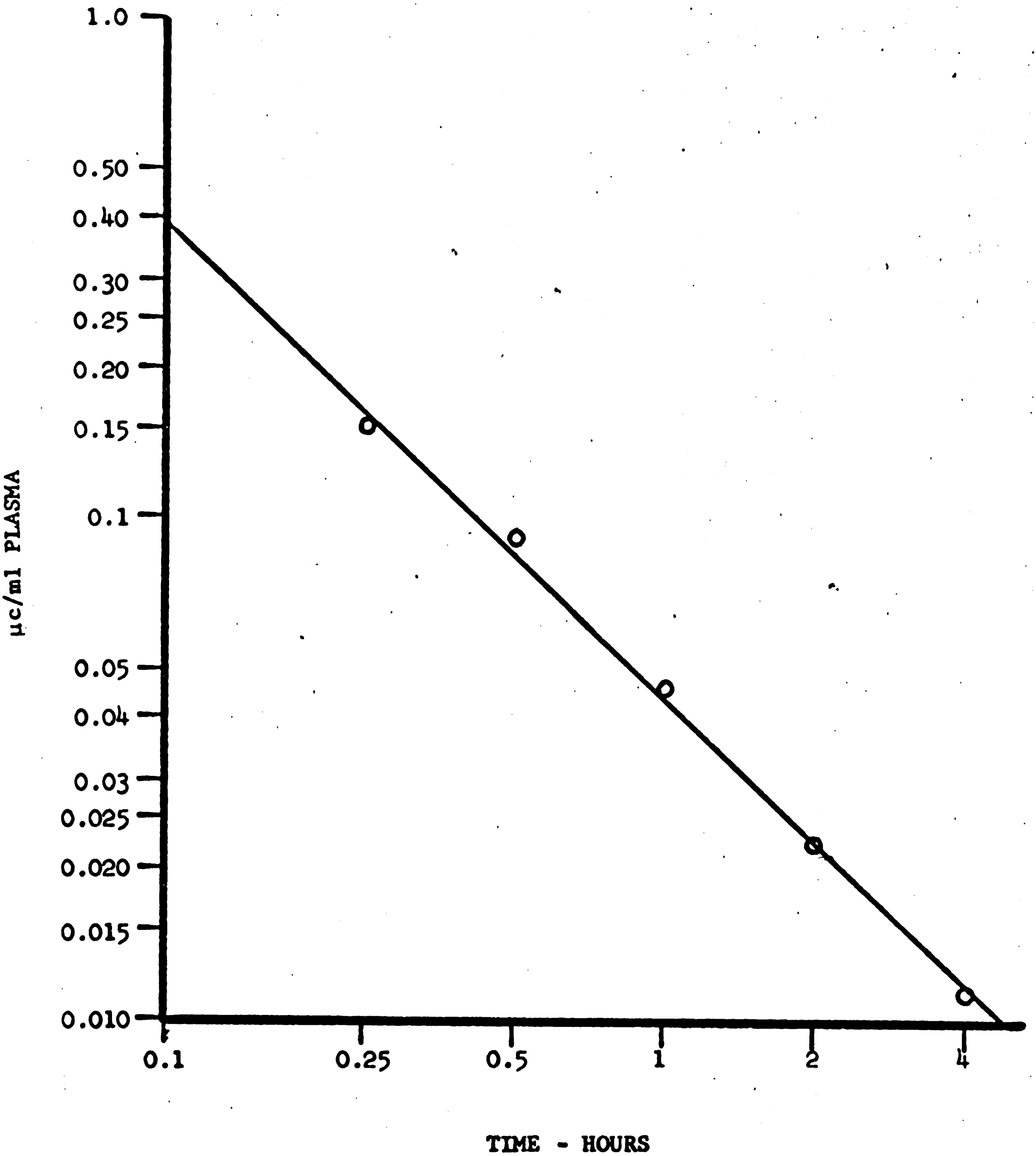


FIGURE 2. CLEARANCE OF H^3-THYMIDINE AND NON-VOLATILE BREAKDOWN PRODUCTS OF H^3-THYMIDINE FROM MOUSE BLOOD PLASMA.

The second experiment to determine the rapidity of uptake of H^3-thymidine in femur bone marrow DNA and whole femur consisted of an injection of either saline or cold thymidine 15 minutes following the H^3-thymidine injection. The protocol for this experiment was the same as outlined in the Materials and Methods, except an injection of 0.2 milliliter of physiological saline or 50 micrograms of cold thymidine per gram of body weight was given 15 minutes after the injection of H^3-thymidine. Figures 3 and 4 show the results of these experiments. There is only a slight increase in activity in the femur and in femur bone marrow DNA between 30 minutes and 1 hour, and in addition, the activity in femur bone marrow DNA only decreased to about 80 percent of the normal after the injection of 1000 micrograms of cold thymidine at 15 minutes. Therefore, most of the labeling must have occurred before 15 minutes. After maximum labeling is achieved, the activity in femur bone marrow DNA of cold thymidine injected animals does not drop. This indicates that the constant activity in femur bone marrow DNA in the normal mouse is not due to a feed-in of H^3-thymidine from the plasma, since plasma tritium is rapidly falling.

The total uptake on the basis of the combusted femur is about 0.04 microcurie/femur. At 4 hours, it would take approximately 3 milliliters of plasma (0.012 microcurie/milliliter) to be equivalent to the activity concentrated in the femur (0.036 microcurie/femur). The loss in plasma activity does not parallel the loss of activity from the femur; the plasma clearance of tritium being much more rapid.

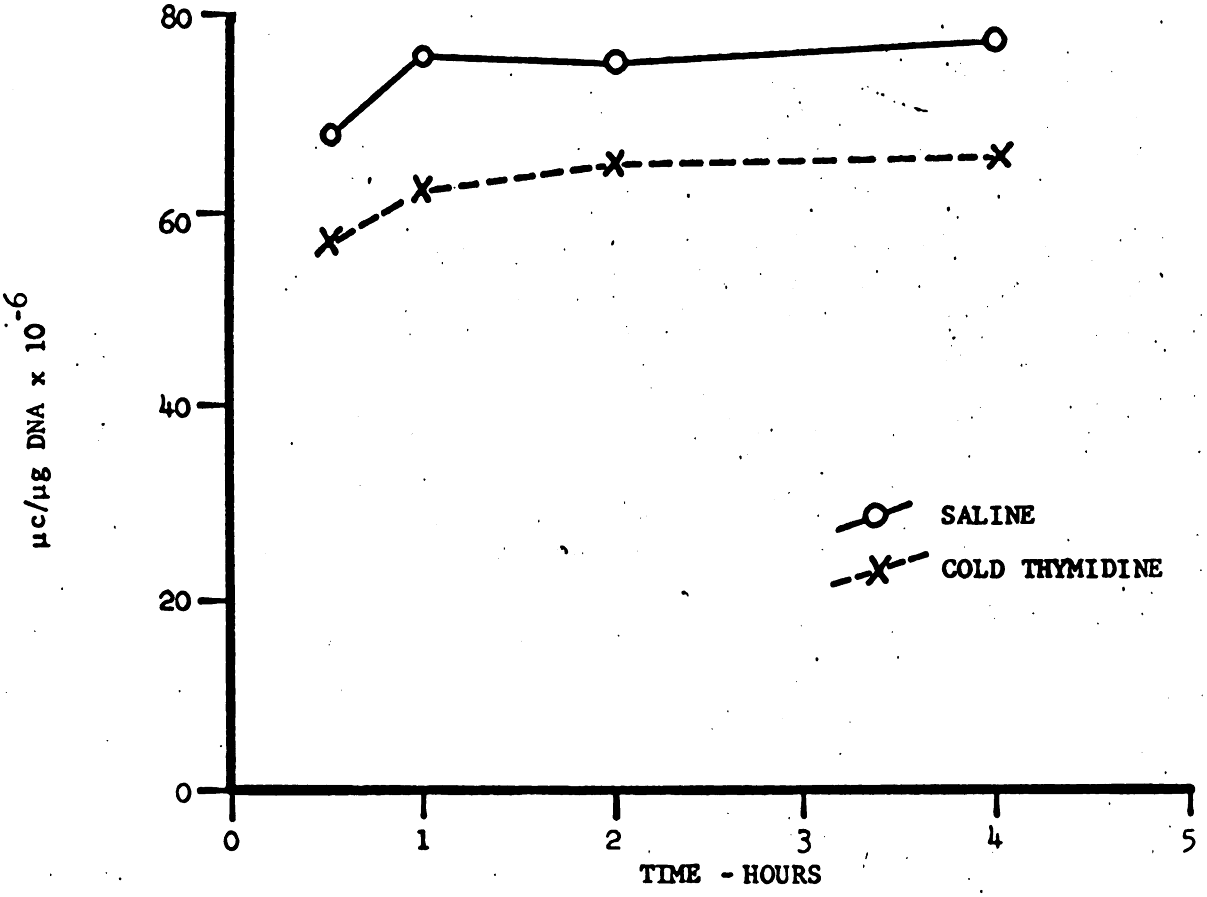


FIGURE 3. FEMUR BONE MARROW DNA ACTIVITY AS A FUNCTION OF TIME. H^3-THYMIDINE WAS INJECTED AT TIME 0 AND SALINE OR COLD THYMIDINE WAS INJECTED AT 15 MINUTES.

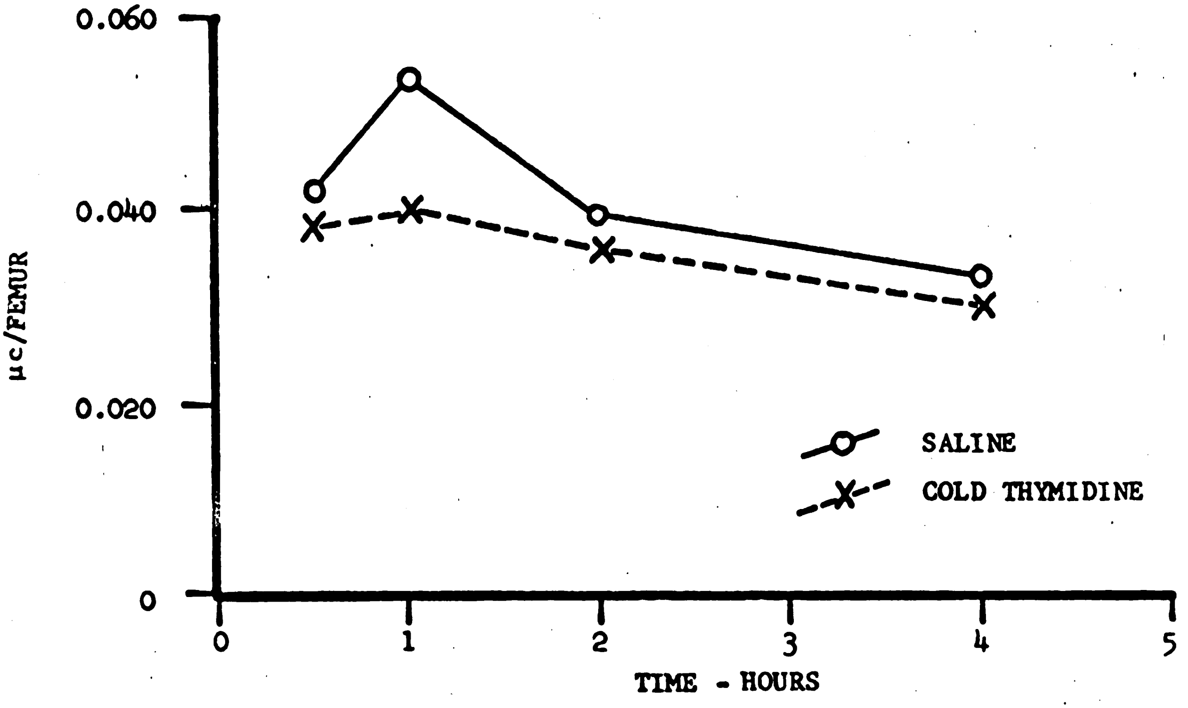


FIGURE 4. WHOLE FEMUR ACTIVITY AS A FUNCTION OF TIME. H^3-THYMIDINE WAS INJECTED AT TIME 0 AND SALINE OR COLD THYMIDINE WAS INJECTED AT 15 MINUTES.

The rapid plasma clearance of non-volatile tritium activity and the subsequent sustained level of tritium activity in the femur and in femur bone marrow DNA indicate that thymidine is incorporated very rapidly.

Activity Incorporated into Femur Bone Marrow Deoxyribonucleic Acid

The activity incorporated into mouse bone marrow DNA was investigated as a function of time. Several individual experiments were run on normal animals. Figure 5 shows the activity in the bone marrow DNA in one femur with time up to 168 hours after H^3-thymidine injection. The activity incorporated in femur bone marrow DNA remains essentially constant for about 18 hours (intercept 69.4×10^{-6} microcurie/microgram DNA -- slope does not differ significantly from zero -- Slope = 0.00508 ± 0.00559). (Table II--Page 43--gives a summary of all pertinent values and their statistics.) Between 18 and 24 hours, additional radioactive DNA is added to the DNA pool in the femoral marrow. The added radioactive DNA must have a much higher activity than was there previously. This is evident when the points after 24 hours are considered. The extrapolated zero time intercept based on these points is 193.7×10^{-6} microcurie/microgram DNA and the slope is -0.01976 ± 0.00190. The twenty-four hour activity is 120.6×10^{-6} microcurie/microgram DNA which is 51.2×10^{-6} microcurie/microgram DNA greater than the previous plateau. This represents an increase in activity of at least 73 percent under steady state conditions

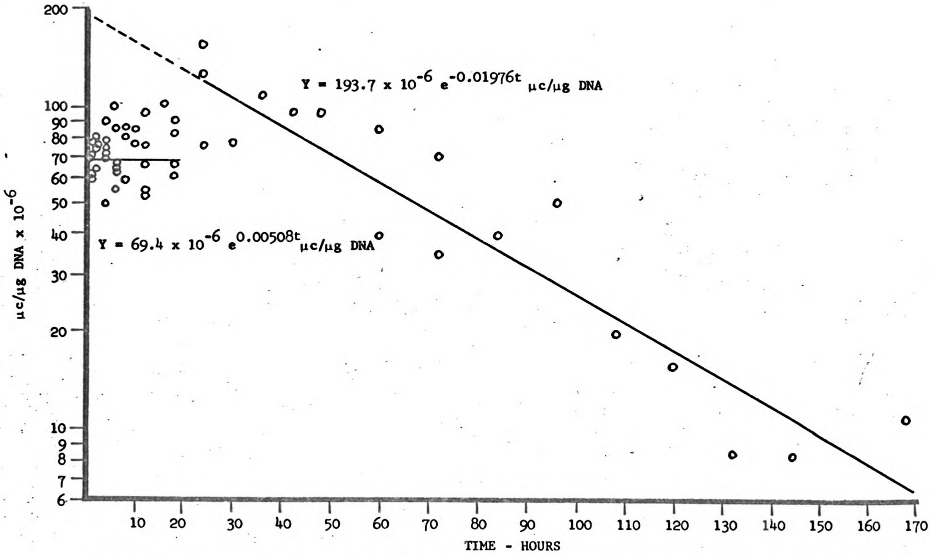


FIGURE 5. CONCENTRATION OF TRITIUM ACTIVITY IN FEMUR BONE MARROW DNA AS A FUNCTION OF TIME FOLLOWING THE ADMINISTRATION OF H^3-THYMIDINE.

where no change in femur bone marrow DNA content per se took place. This could only occur if after 18 hours, but before 24 hours, some highly labeled DNA precursor was actually incorporated into the extracted DNA itself, or alternatively, if high activity DNA, preformed elsewhere, was moved into the femur as part of a steady state system. In either case, there would seem to be at least two ways for H^3-thymidine to enter the femur bone marrow DNA pool, at least into that moiety which is extracted as the total DNA of the marrow of the femur. This point will be pursued further in subsequent sections.

It is of interest to compare these results with Steel's data for rats (Steel, 1962), Figure 6. If one takes LoBues' figure (LoBue et al., 1963) for the number of cells per gram of rat bone marrow (2×10^9 cells/gram) and the figure obtained from these experiments for the amount of DNA per cell (8.9×10^{-6} microgram DNA/cell -- See Appendix V), Steel's data can be put on the same basis as the results presented in Figure 5. Figure 6 is a plot of his data, recalculated. The intercept is the same (74.8×10^{-6} microcurie/microgram DNA versus 69.4×10^{-6} microcurie/microgram DNA) and the activity likewise remains constant for the first part of the curve. The half time from his rat data after 24 hours is 1.6 days; for these mouse experiments it is 1.5 days.

Steel's data were obtained following the injection of H^3-thymidine with a specific activity of 1.9 curies/millimole. This is only one-third the specific activity used in the present work. However, the same amount of activity (0.5 microcurie per gram body weight)

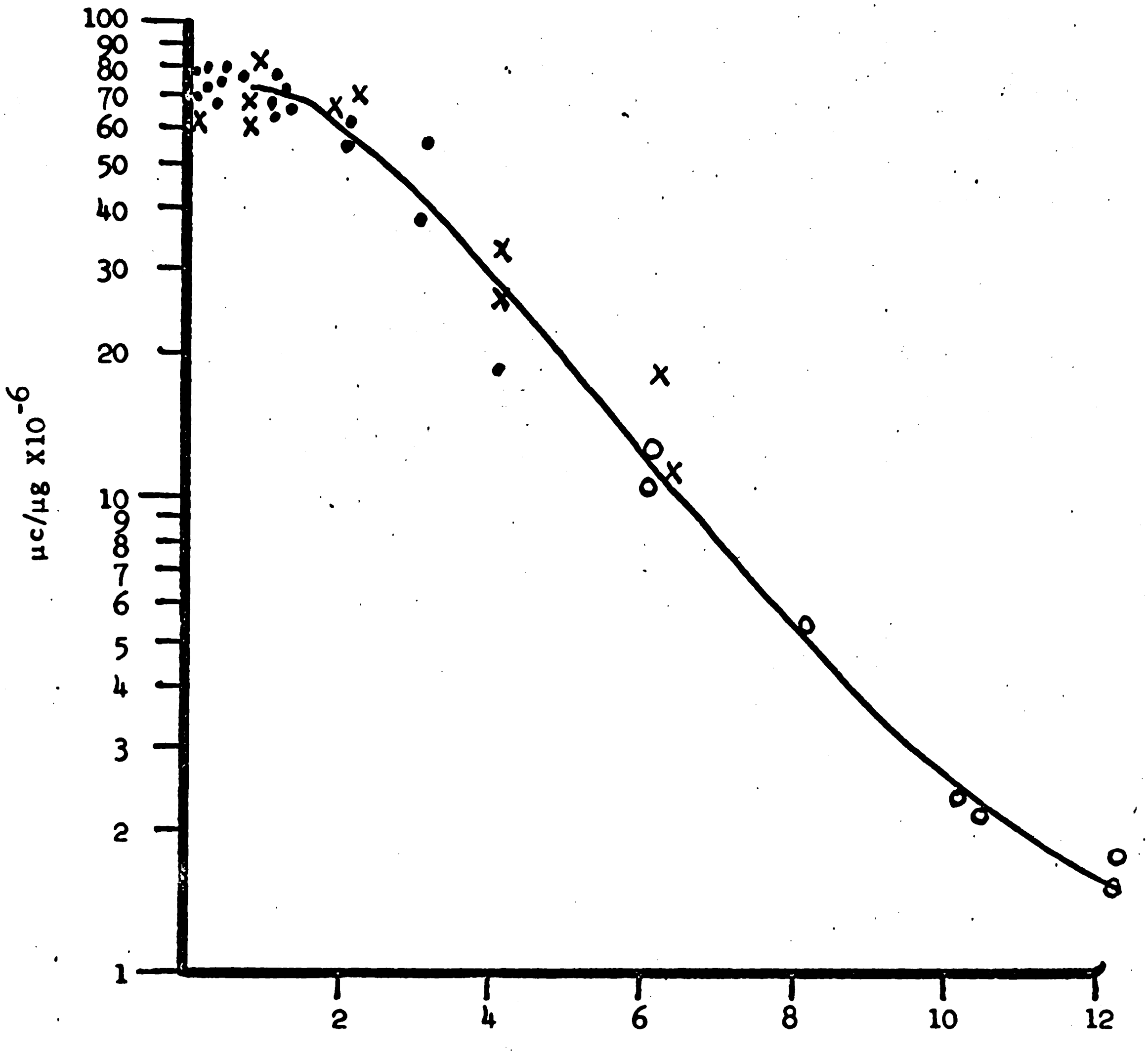


FIGURE 6. THE CONCENTRATION OF NON-VOLATILE TRITIUM ACTIVITY IN NORMAL BONE MARROW AFTER AN INJECTION OF H^3-THYMIDINE. THE RESULTS OF THREE EXPERIMENTS ARE COMBINED WITH A DIFFERENT SYMBOL FOR EACH. (AFTER STEEL, 1962)

was injected. Therefore, there must be a large pool of endogenous precursor which dilutes the labeled material so that a true tracer experiment is obtained even with the lower specific activity material.

However, the most important feature of Steel's procedure is that his data were obtained from fat-free combusted bone marrow cells. That is, his method did not involve extraction of DNA from bone marrow cells *per se*, but the combustion of dried cells in the same fashion as the femur data described in this work. Since his data give the same type of curve as one finds after analyzing extracted femur bone marrow DNA, the implication is that any tritium activity not incorporated into DNA must have been washed out by his fat extraction procedure. This is presumably true also in processing autoradiographs since very few grains are observed over the cytoplasm. Therefore, if any non-DNA tritium was present in cells or interstitial spaces in the femur, it would not be observed after chemical DNA extraction techniques, after fat free cell analysis, or after autoradiography.

Activity Incorporated in Whole Femur

The average activity recovered from the combusted whole femurs at each time interval from ten experiments on normal mice (see Appendix VI - Table VI for individual experiments) is presented in Figure 7. Since these results are based on combusted, whole femurs, all of the activity except that from volatile tritium was measured. Figure 7 shows that the activity in the femur starts to decrease (intercept 1-18 hours, 0.0458 microcurie/femur, slope = -0.02017 ± 0.00308 per hour) at 1-2 hours and falls by approximately

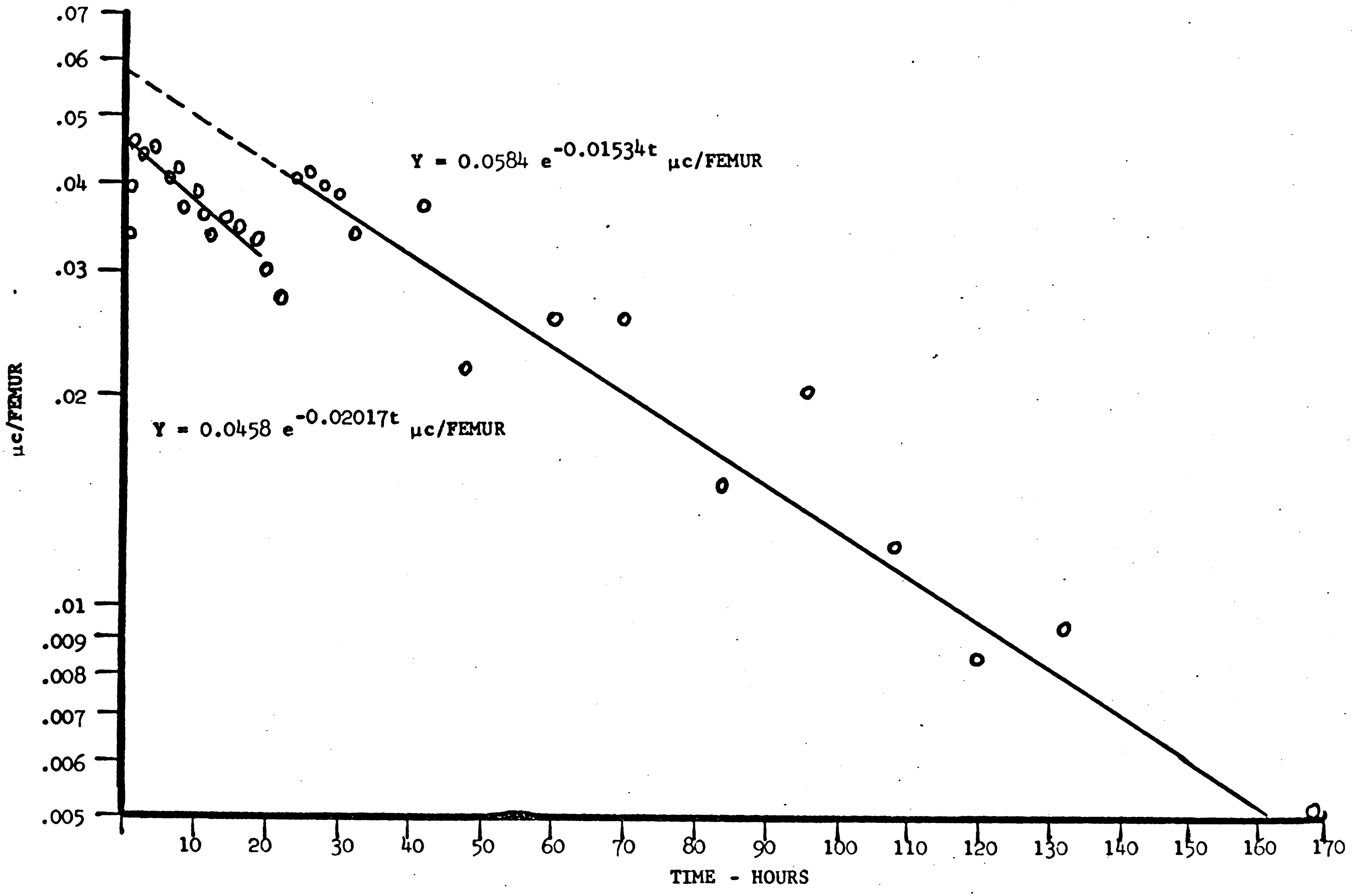


FIGURE 7. TRITIUM ACTIVITY INCORPORATED IN THE WHOLE FEMUR (NORMAL ANIMALS) AS A FUNCTION OF TIME FOLLOWING THE ADMINISTRATION OF H^3-THYMIDINE.

30 percent in 18 hours. This loss does not parallel the fall in plasma activity, which is much more rapid. If the change in non-volatile tritium activity of the combusted femur represents the activity in DNA, it must be reconciled with the activity actually found in femur bone marrow DNA. The measured femur bone marrow DNA activity, however, did not change during the first 18 hours. The explanation for this difference will be considered in the Discussion.

Between 18 and 24 hours, there is an increase in femur activity of about 1.3 times the 18 hour value (0.0317 microcurie/femur to 0.0410 microcurie/femur). This is not as great a relative increase as that observed in the femur bone marrow DNA activity.

When the points after 24 hours are considered, the intercept is 0.0584 microcurie/femur with a slope of -0.01534 ± 0.00102 per hour. There is no significant difference between the slopes of the activity in femur bone marrow DNA after 24 hours (Figure 5) and the activity in the femur after 24 hours (Figure 7). This indicates that the activity determined on the basis of non-volatile tritium activity in the femur must be in femur bone marrow DNA after 24 hours, but not completely so before 24 hours.

Bone Marrow Cell DNA Content Per Femur Estimated From the Activity in DNA and Activity in Femur

Since the femur activity after 24 hours represents bone marrow cell DNA activity, the total amount of bone marrow cell DNA per femur can be estimated by taking the extrapolated intercepts based on the points after 24 hours. From Figures 7 and 5, the values are 0.0584 microcurie/femur and 193.4×10^{-6} microcurie/microgram DNA respectively. Thus, the amount of DNA per femur is:

$$\frac{0.0584\ \mu c/\text{femur}}{193.4 \times 10^{-6}\ \mu c/\mu g\ \text{DNA}} = 300\ \mu g\ \text{DNA/femur}.$$

It is possible to convert this value into the number of cells per femur. In the course of the DNA determinations on femur bone marrow cells, an analysis of the amount of DNA per cell was made. The figure obtained (8.9×10^{-6} microgram DNA/cell) agrees well with values in the literature (Davidson, Leslie and White, 1951; Thomson _et_ _al_., 1953). The number of cells per femur based on 300 micrograms DNA/femur is:

$$\frac{300\ \mu g\ \text{DNA/femur}}{8.9 \times 10^{-6}\ \mu g\ \text{DNA/cell}} = 3.4 \times 10^{7}\ \text{cells/femur}.$$

Figure 8 is a plot of the DNA activity put on the basis of the whole femur, i.e., the activity in DNA in Figure 5 has been multiplied by 300 micrograms DNA/femur.

Activity Incorporated into the Whole Femur - Hypertransfused Mice

Data on the activity in femur bone marrow DNA are not available for hypertransfused animals, however, the data obtained from femur combustion (non-volatile tritium activity) can be evaluated on the basis of the change in the differential cell count of bone marrow between normal and polycythemic mice, if the DNA pool size is assumed to be the same. A comparison of the bone marrow differentials between normal and hypertransfused mice is shown in Table I.

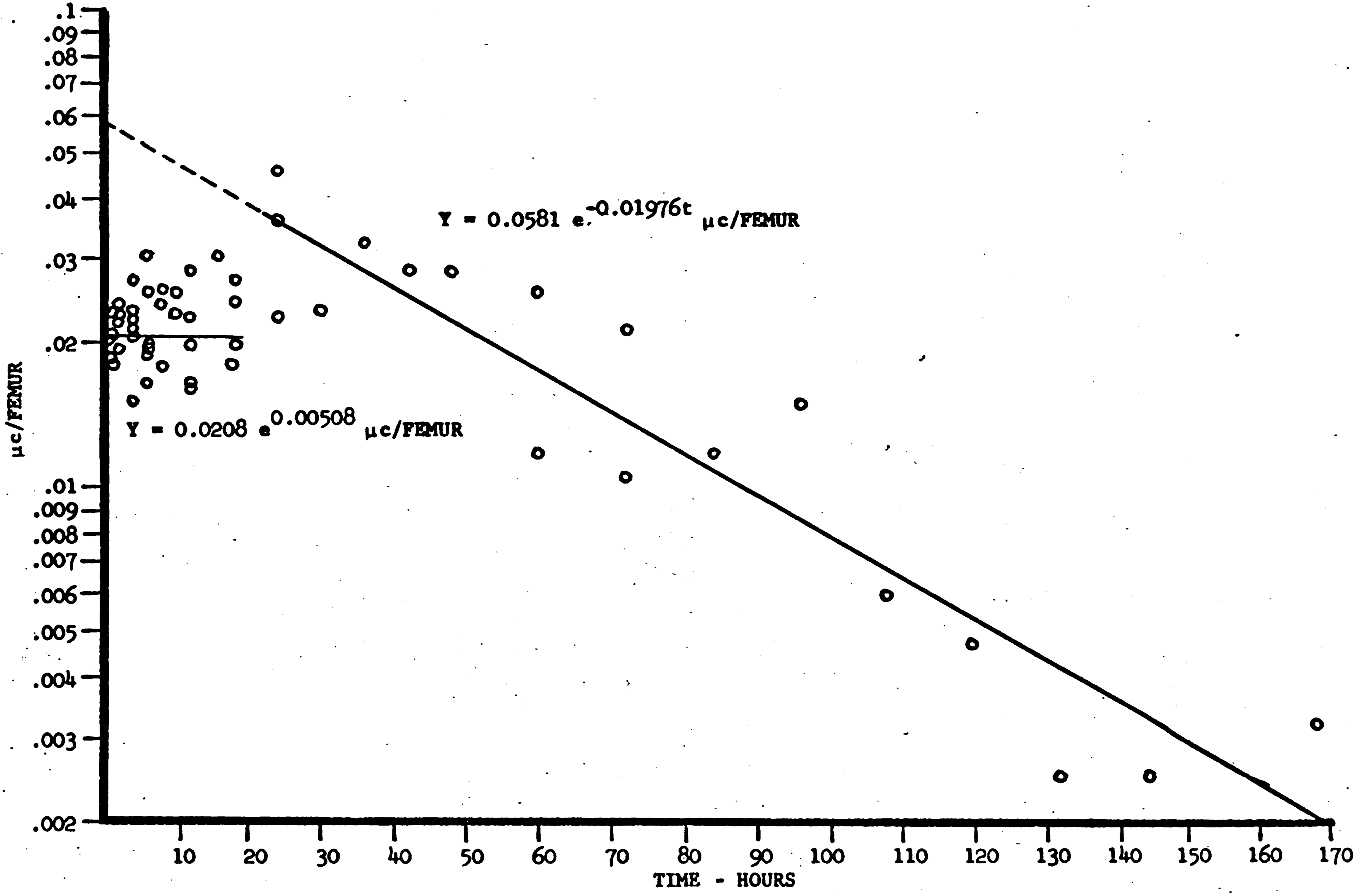


FIGURE 8. FEMUR BONE MARROW CELL DNA ACTIVITY INCORPORATED PER FEMUR AS A FUNCTION OF TIME AFTER A SINGLE INJECTION OF H^3-THYMIDINE.

TABLE I

DISTRIBUTION OF CELLS OR DNA IN THE FEMUR OF NORMAL AND HYPERTRANSFUSED MICE

Cell Type	Normal			Hypertransfused		
	Differential (% Total)	μg DNA (Total = 300μg)	Number of Cells* (Total = 3.4×10^7)	Differential (% Total)	μg DNA (Total = 300μg)	Number of Cells* (Total = 3.4×10^7)
Myeloblast	1.3	3.9	0.44×10^6	2.6	7.8	0.88×10^6
Promyelocyte	1.9	5.7	0.64×10^6	3.7	11.1	1.26×10^6
Myelocyte	10.3	30.9	3.47×10^6	20.6	61.8	7.00×10^6
Metamyelocyte	11.6	34.8	3.91×10^6	21.7	65.1	7.38×10^6
Band	9.0	27.0	3.03×10^6	19.1	57.3	6.49×10^6
Poly	5.2	15.6	1.75×10^6	5.1	15.3	1.87×10^6
Lymphocyte	25.2	75.6	8.50×10^6	20.6	61.8	7.00×10^6
Red Proliferative	18.1	54.3	6.10×10^6	3.7	11.1	1.26×10^6
Red Non-Proliferative	17.4	52.2	5.86×10^6	2.8	8.4	0.95×10^6

* using 8.9×10^{-6} μg DNA/cell - See Appendix V

Table I indicates that the overall effect of hypertransfusion is to cut down red cell production and expand the granulocyte compartment. The increase in size of the granulocyte compartment is noted also in the total number of cells obtained from the femur of hypertransfused animals. Although under the experimental conditions used, it is not possible to quantitate the cell number obtained from a femur, the technique of removing the marrow plug with positive pressure gives fairly consistently 1 x 10^7 cells in normal mice, or about 30 percent of the total cells in the femur. In several experiments with hypertransfused animals about 1 x 10^7 cells were obtained also. Therefore, as a first approximation, the DNA pools of normal and hypertransfused animals may be considered to be equal.

Figure 9 represents the average of 4 experiments on hypertransfused animals (see Appendix VI - Table VII for individual experiments). The data show that there is no significant difference between the initial uptake (1-18 hours) in the hypertransfused animal (intercept 0.0458 microcurie/femur) and the initial uptake in the normal animal (Figure 7). Between 18 and 24 hours, an increase in activity amounting to 1.3 times the activity at 18 hours is observed. This is the same increment that is observed in the normal animal (Figure 7). When the points after 24 hours are considered, there is no significant difference between the extrapolated intercepts of hypertransfused animals (0.0560microcurie/femur -- Figure 9), the normal animal

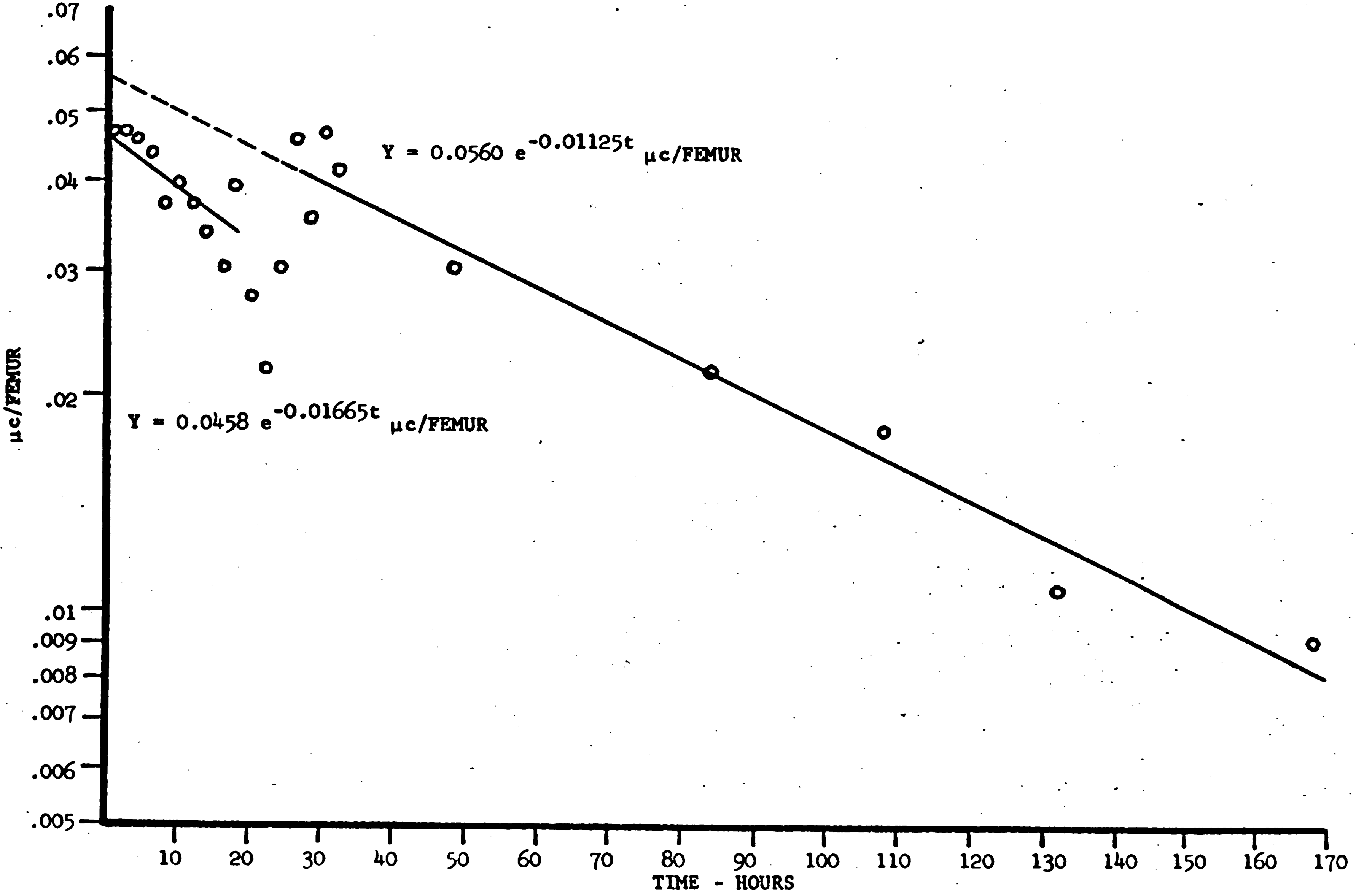


FIGURE 9. TRITIUM ACTIVITY INCORPORATED IN THE WHOLE FEMUR (HYPERTRANSFUSED ANIMALS) AS A FUNCTION OF TIME AFTER A SINGLE INJECTION OF H^3-THYMIDINE.

(0.0584 microcurie/femur -- Figure 7), and the femur activity in the normal animal estimated from DNA activity (0.0581 microcurie/femur -- Figure 8). It is reasonable for the initial and final activities to be the same only if the DNA pool size is constant, and the numbers of proliferative cells are the same. From Table I, one can see that the overall percentages of cells in the proliferative compartment (red proliferative, myeloblast, promyelocyte, and myelocyte) are not significantly changed between normal and hypertransfused animals (31.6 percent vs. 30.8 percent).

When the points after 24 hours are considered, there is a significant difference in slopes of Figure 7 (normal mice) and Figure 9 (hypertransfused mice). Because of this, the calculated output of femur bone marrow DNA in hypertransfused animals is about 2/3 that of the normal animals (Table II). Since the granulocyte compartment in the hypertransfused animal is twice that of the normal animal (Table I), the DNA output of the hypertransfused animals represents 2 times the normal granulocyte DNA output plus the lymphocyte DNA; whereas the DNA output in the normal animal represents the DNA output of granulocytes, lymphocytes and red cells. Therefore, the slower output in the hypertransfused animal means that the DNA output in the normal animal must be much greater than the DNA output from the granulocytes in the normal animal, since the pool sizes appear to be equal. This is true only if the cell cycle time, the number of cell divisions, the extent of cell loss occurring in the granulocyte compartment, and the turnover of other DNA components (e.g., lymphocytes) remain constant. This point will be considered in more detail in the Discussion.

Granulocyte and Lymphocyte Appearance in the Peripheral Blood

Autoradiographs were prepared on slides of peripheral blood from both control and hypertransfused animals. Figure 10 shows the mean grain count per cell for all granulocytes (labeled and unlabeled) and Figure 11 shows the mean grain count per cell for all lymphocytes (labeled and unlabeled). These results are presented as mean grain count per cell rather than percent labeled to emphasize the difference in activity per cell between lymphocytes and granulocytes. The early loss in femur activity (Figures 7 and 9), is not due to the loss of granulocytes to the blood in either normal or hypertransfused mice since labeled granulocytes do not appear in the peripheral blood until 24 hours. This work confirms the work of Bryant and Kelly (1958) who used carbon-14 labeled adenine to study the appearance of lymphocytes and granulocytes in the peripheral blood.

Summary of Pertinent Data and Their Statistics

Table II presents a summary of the intercepts and their errors, the slopes and their errors, the rate constants, the half-times, and the output in micrograms of DNA per hour from the activity in femur bone marrow DNA and femur activity in each experiment at the times indicated in the Results.

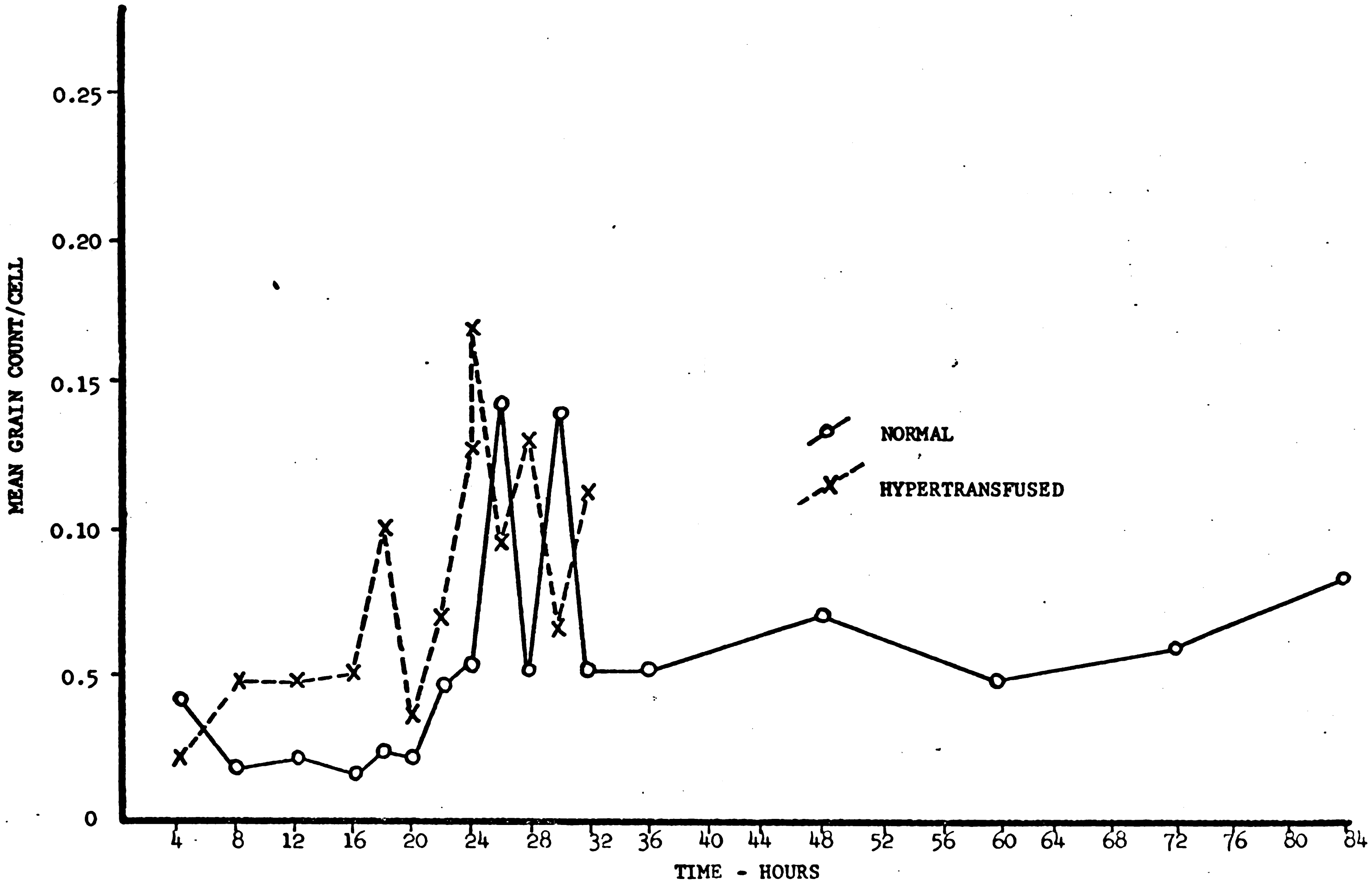


FIGURE 10. GRANULOCYTE LABELING IN THE PERIPHERAL BLOOD OF NORMAL AND HYPERTRANSFUSED MICE GIVEN A SINGLE INJECTION OF H^3-THYMIDINE.

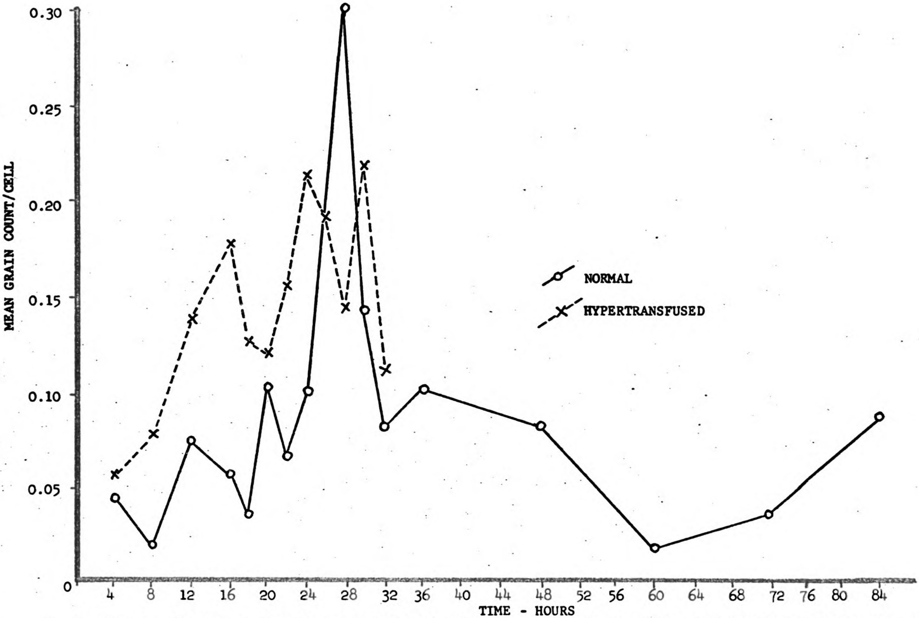


FIGURE 11. LYMPHOCYTE LABELING IN THE PERIPHERAL BLOOD OF NORMAL AND HYPERTRANSFUSED MICE GIVEN A SINGLE INJECTION OF H^3-THYMIDINE.

TABLE II

CONSTANTS OF LEAST SQUARES LINES AND DNA OUTPUT - FIGURES 5, 7 and 9

	Intercept	ln Intercept ± Standard Error	Slope ± Standard Error (hours^{-1})	Half Time (hours)	DNA Output per femur* (µg DNA/hour)
Normal Animals DNA Activity 1-18 hours Figure 5	69.4×10^{-6} µc/µg DNA	-9.56492 ± 0.05094	0.00508 ± 0.00559	--	--
Normal Animals DNA Activity 24-168 hours Figure 5	193.7×10^{-6} µc/µg DNA	-8.53956 ± 0.01642	-0.01976 ± 0.00190	35.1	5.93
Normal Animals Femur Activity 1-18 hours Figure 7	0.0458 µc/Femur	-3.07892 ± 0.03087	-0.02017 ± 0.00308	34.4	6.05
Normal Animals Femur Activity 24-168 hours Figure 7	0.0584 µc/Femur	-2.83776 ± 0.09485	-0.01534 ± 0.00102	45.2	4.60

TABLE II (Continued)

	Intercept	ln Intercept ± Standard Error	Slope ± Standard Error (hours^{-1})	Half Time (hours)	DNA Output per femur* (μg DNA/hour)
Hypertransfused Animals **Femur Activity** **1-18 hours** **Figure 9**	0.0458 μc/Femur	-3.07676 ± 0.05902	-0.01665 ± 0.00083	41.6	5.00
Hypertransfused Animals **Femur Activity** **24-168 hours** **Figure 9**	0.0560 μc/Femur	-2.87875 ± 0.07227	-0.01021 ± 0.000819	61.6	3.06

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*Obtained by using a pool size of 300 μg DNA/femur.

DISCUSSION

The principal events occurring between an intravenous injection of H^3-thymidine and the observation of labeled bone marrow DNA in the femur are shown in Figure 12. During the period of availability, all cells in DNA synthesis are capable of incorporating labeled nucleoside. In addition, it may be possible for the labeled precursor to be shunted into a storage pool to be used later in DNA formation. Since the DNA pool size is constant, DNA incorporated activity can be altered only by DNA turnover (cell turnover), feed-in from a labeled substrate pool, or reutilization of labeled breakdown products of DNA.

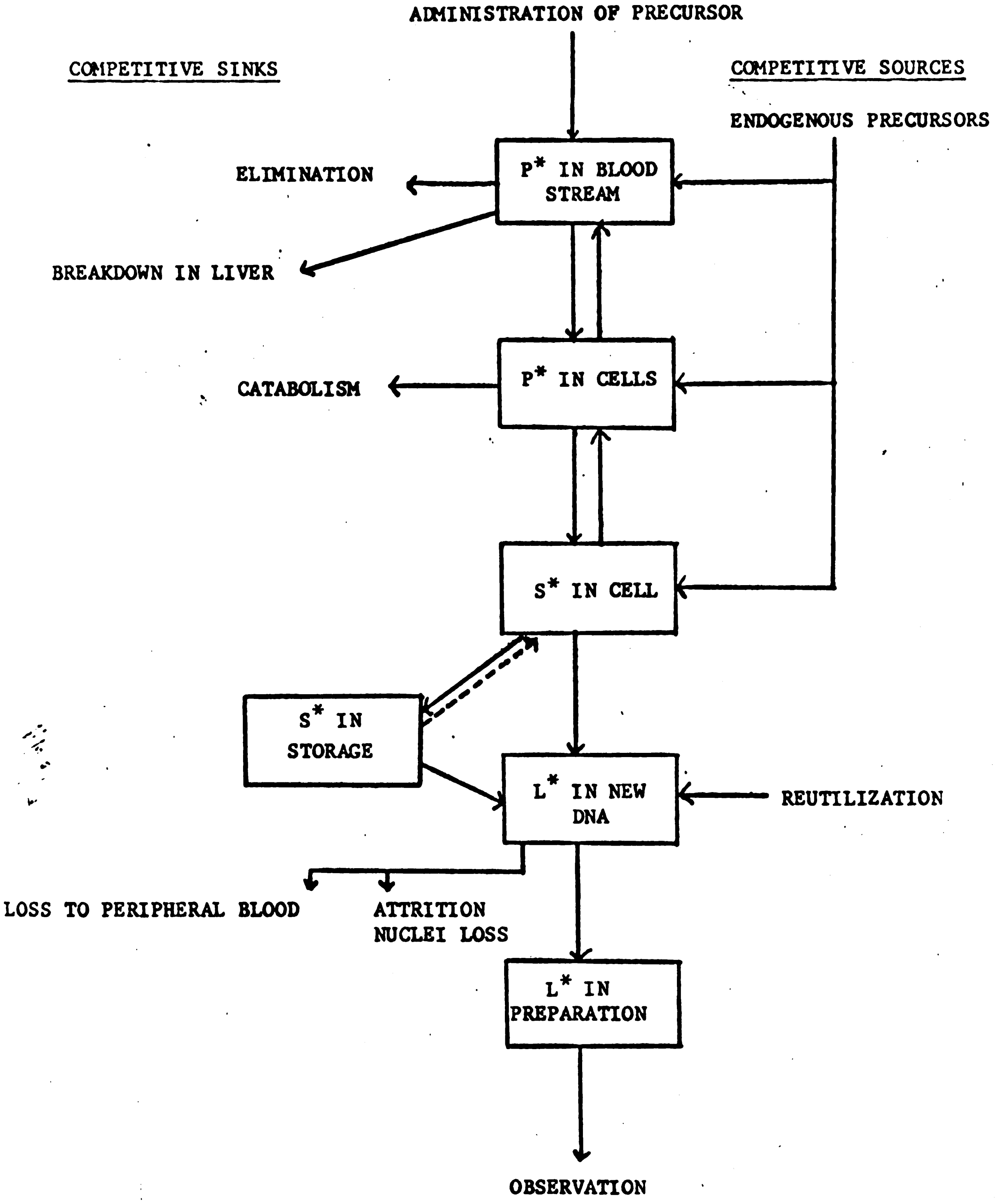
In these experiments an examination has been made of H^3-thymidine incorporation into a tissue which is known to be undergoing rapid DNA production. From the description of cell renewal systems, and of bone marrow cell proliferation in particular, outlined in the Introduction, it is possible to make certain generalizations about tritium exchange in the bone marrow DNA pool of the femur after initial labeling of DNA is complete:

Input = 1) Tritium labeled DNA from the stem cell (if the stem cell is outside femur). The stem cell input is considered to be low (Patt and Maloney, 1963).

2) Tritium labeled DNA from lymphocyte turnover.

3) Tritium labeled DNA from unknown sources (reutilization).

4) Tritium labeled substrate from storage pool.



P^* - LABELED THYMIDINE S^* - LABELED SUBSTRATE L^* - LABELED DNA

FIGURE 12. THE MOVEMENT OF LABELED TRITIUM COMPOUNDS IN THE FEMUR BONE MARROW CELLS AFTER A SINGLE INJECTION OF TRITIATED THYMIDINE. THIS DIAGRAM COULD APPLY EQUALLY WELL TO OTHER CELL RENEWAL SYSTEMS.

Output = 1) Tritium labeled DNA from possible granulocyte attrition.

2) Tritium labeled DNA from erythrocyte nuclei loss.

3) Tritium labeled DNA from lymphocyte turnover.

4) Tritium labeled DNA from granulocyte loss to the blood.

Analysis of femur DNA will also reflect DNA activity in such cells as megakaryocytes and osteoblasts. The change in DNA activity from these sources is assumed to be low in comparison to the changes in DNA activity occurring in the erythroid and myeloid compartments of the femur.

Difference in Femur Bone Marrow Deoxyribonucleic Acid Activity and Femur Activity

The data obtained from femur bone marrow DNA activity (Figure 8) and femur activity (Figure 7) are different during the first 18 hours in that the femur bone marrow DNA activity remains constant (0.0208 microcurie/femur) while the femur activity drops by about 30 percent of its initial value (0.0458 microcurie/femur to 0.0317 microcurie/femur). Since the bone marrow cell population represents a cell renewal system, the DNA incorporated activity should decrease with time, unless there is input of activity. It is not possible to explain the constant activity in femur bone marrow DNA over the first 18 hours on the basis of a long initial period of availability of H^3-thymidine in the blood.

Reutilization of breakdown products of femur bone marrow DNA might possibly account for the constant activity in femur bone marrow DNA during the first 18 hours. However, on the basis of the uptake in the femur (0.0458 microcurie /femur) the specific activity of the material injected (6 curies/millimole) is diluted about 500 times. [There are 63 micrograms of bone marrow thymidine per femur (300 micrograms DNA/femur x 0.21 microgram thymidine/microgram DNA), of which about 1/3 are in proliferative cells (Table I). When DNA synthesis occurs, 1/2 of the new DNA contains new thymidine. The total thymidine incorporated into the femur along with the 0.0458 microcurie by this calculation would be 10.5 micrograms. The specific activity of the incorporated material would then be 0.0046 microcurie/microgram of thymidine, as compared to the 6 curie/243 milligram of thymidine injected.] This is only an estimate, but there is evidently a great dilution of the injected material.

Since labeled lymphocytes are seen in the peripheral blood at early times, and lymphocytes represent about 25 percent of the total femur marrow (Table I) it is possible that labeled lymphocytes could be preferentially taken up and thus keep the activity in femur bone marrow DNA constant. A recent report by Osmond and Everett (1964) indicates that this is unlikely. They show in mice, that after a single injection of H^3-thymidine, the bone marrow lymphocyte labeling index increases to about 40 percent in 3 days. If, however, they occlude circulation to one hind limb and administer H^3-thymidine, then restore circulation 20 minutes later, no labeled lymphocytes accumulate in the occluded limb. Labeling, however, proceeds in the normal fashion in the control limb. They maintain that no pathological changes took place in the limb which had its circulation occluded since the distribution of lymphocytes between the normal and control limbs remains the same throughout the experimental period. These authors conclude that lymphocytes are not entering the bone marrow from the peripheral blood, and raise the question of intramedullary lymphocytopoiesis. If there is intramedullary lymphocytopoiesis, then the possibility of there being a labeled substrate pool at the tissue level arises since the labeling index continues to increase for 3 days after the injection of H^3-thymidine.

A long availability time of labeled precursor in the blood, reutilization of labeled breakdown production from femur bone marrow DNA and other sites of cell renewal, or a labeled lymphocyte feed-in to the marrow do not seem to explain the constant level in femur bone marrow DNA activity while the femur activity drops.

If any one of these alternatives was accepted to explain the constant femur bone marrow DNA activity, it would imply that the observed drop in femur activity is due to non-volatile tritium clearance. It was pointed out in the Results that the drop in femur activity was not due to the plasma clearance. Since the activity in femur bone marrow DNA should drop with time, then it is necessary to examine the femur drop for the possibility of this being, in fact, a drop in femur bone marrow DNA activity.

If the initial uptake of label into DNA was distributed proportionally between erythrocyte and granulocyte proliferative cells, then the red cell precursors would have 57 percent of the label or 0.0119 microcurie/femur and the granulocyte precursors would have 43 percent of the label or 0.0089 microcurie/femur (see Table I). The rate of loss of label due to the loss of the red cell nucleus can be evaluated in the following way:

Assumptions:

Number of circulating red blood cells = 8×10^9 cells/milliliter

Blood volume of a mouse = 7 percent of body weight

Life span of a mouse red blood cell = 42 days

Single femur marrow volume = 5 percent of total marrow volume (LoBue, 1963)

Therefore, the total number of red blood cells produced by the bone marrow of one femur in a 20 gram mouse is:

$$= \frac{(8 \times 10^9)\ (0.07)\ (20)}{(42)\ (24)\ (20)}$$

$= 0.55 \times 10^6$ cells/hour/femur.

There are 12×10^6 nucleated erythrocyte precursors per femur (see Table I). Therefore, the turnover constant for the red cell precursors would be:

$$\frac{0.55 \times 10^6}{12 \times 10^6} = 0.046/\text{hour}. \quad (\text{Half-Time} = 15 \text{ hours}).$$

This should also be the rate at which the label is being lost. Therefore, in 18 hours, 0.0067 microcurie/femur will be lost. $(0.0119 - 0.0119e^{(-0.046)\ (18)})$.

The loss in femur activity in the hypertransfused animal in 18 hours was 25.6 percent (Figure 9). This loss should be due to granulocyte attrition, since mature granulocytes were not lost to the blood until 24 hours (Figure 10), and red cell production was entirely suppressed. If the uptake in hypertransfused mouse femur bone marrow DNA is the same as in the normal animal (0.0208 microcurie/femur), then the total loss from bone marrow DNA would be 0.0089 microcurie in 18 hours: (0.0458 x 0.256 = 0.0119 microcurie/femur and 0.0208 - 0.0119 = 0.0089 microcurie/femur).

As pointed out in the Results, the loss due to granulocyte attrition in the hypertransfused animal is about twice that of the normal animal. Therefore, the total loss due to granulocyte attrition in the normal animal would be 0.0044 microcurie/femur (0.0089/2).

The total loss in 18 hours in the normal animal would be:

Loss of red cell nucleus = 0.0067 microcurie/femur
Loss of granulocytes = 0.0044 microcurie/femur
Total activity lost = 0.0111 microcurie/femur.

This is a total loss in femur activity in 18 hours of 24 percent. The observed loss was 0.0141 microcurie/femur or 31 percent (Figure 7).

It is important to estimate the time of expected loss in femur bone marrow DNA activity. Labeled mitoses have been observed as early as 1/2 hour after an injection of H^3-thymidine (Patt, 1959), thus in the case of attrition, the loss of labeled cells could presumably occur at any time after the completion of cell division.

The time to enucleation of non-proliferative erythrocytic precursors is about 10 hours (estimated from red cell production of 0.55×10^6 cells/hour/femur and the number of non-proliferative red cell precursors of 5.86×10^6 cells/femur - Table 1). The loss of nuclear material from erythrocyte precursors may be a gradual process extending over the life of the orthochromatic normoblast, rather than an abrupt loss of the nucleus. This would mean that the loss of activity from DNA would be a gradual process that might begin after the last cell division. A calculation of this type should be considered only a first approximation, since many assumptions are made which may be in error. It does serve to indicate that an early loss in femur bone marrow DNA activity is not an unexpected phenomenon.

In view of the preceding discussion, and the extended labeling period of the bone marrow lymphocyte reported by Osmond and Everett, and the labeling of nucleated erythroid precursors in erythropoietin stimulated hypertransfused mice reported by Schooley and Giger (1962) and Gurney (1962) it seems that consideration should be given to examining the possibility of an extended labeling period of DNA after a single injection of H^3-thymidine.

Consideration of a "Pre-DNA" Pool Feeding Labeled Substrate into Femur Bone Marrow Deoxyribonucleic Acid

It is possible to reconcile the differences between femur bone marrow DNA activity and femur activity during the first 18 hours if a pool feeding labeled substrate into DNA is postulated. A pool of this sort is represented by the difference between the activity in femur bone marrow DNA from 1-18 hours and the total activity in the femur between 1-18 hours. The DNA activity before 18 hours is 0.0208 microcurie/femur (Figure 8) and the femur activity is 0.0458 microcurie/femur (Figure 7). This difference is represented by:

$$\text{Non-DNA activity} = \text{Total activity} - \text{DNA activity}$$
$$= 0.0458\ e^{-0.02107t} - 0.0208\ e^{0.00508t}$$

At time 0 an extrapolated value for the total non-DNA activity assumed to represent "Pre-DNA activity" would be 0.0250 microcurie/femur. This would be equivalent to 129 µg of "pre-DNA units"* $\left(\frac{0.0250\ \text{microcurie/femur}}{193.4 \times 10^{-6}\ \text{microcurie /microgram DNA}}\right)$. A semi-log plot of the difference between total femur activity and femur bone marrow DNA activity with time is essentially linear for 18 hours. The half-time of the "pre-DNA pool" is 15 hours and its rate constant 0.0462/hour. The output of "pre-DNA units" then is 5.9 micrograms/hour and the output in femur bone marrow DNA is 5.9 micrograms DNA/hour (see Table II). Although these outputs are the same, these values should be considered to have at least a 10 percent error (5.4 to 6.5 micrograms/hour on the basis of the femur bone marrow DNA slope after 24 hours). If these outputs are equal, the activity in femur bone marrow DNA will remain constant as long as this pool (pre-DNA pool) supplies labeled precursor to DNA. It should be pointed out that the output of the pre-DNA pool has been determined independently of the DNA output, (i.e., the figures for the determination of the pre-DNA pool output were not used in the determination of the DNA output). A model of total femur activity, and the pre-DNA pool activity, along with the inflows and outflows of other contributions to the activity are shown in Figure 13.

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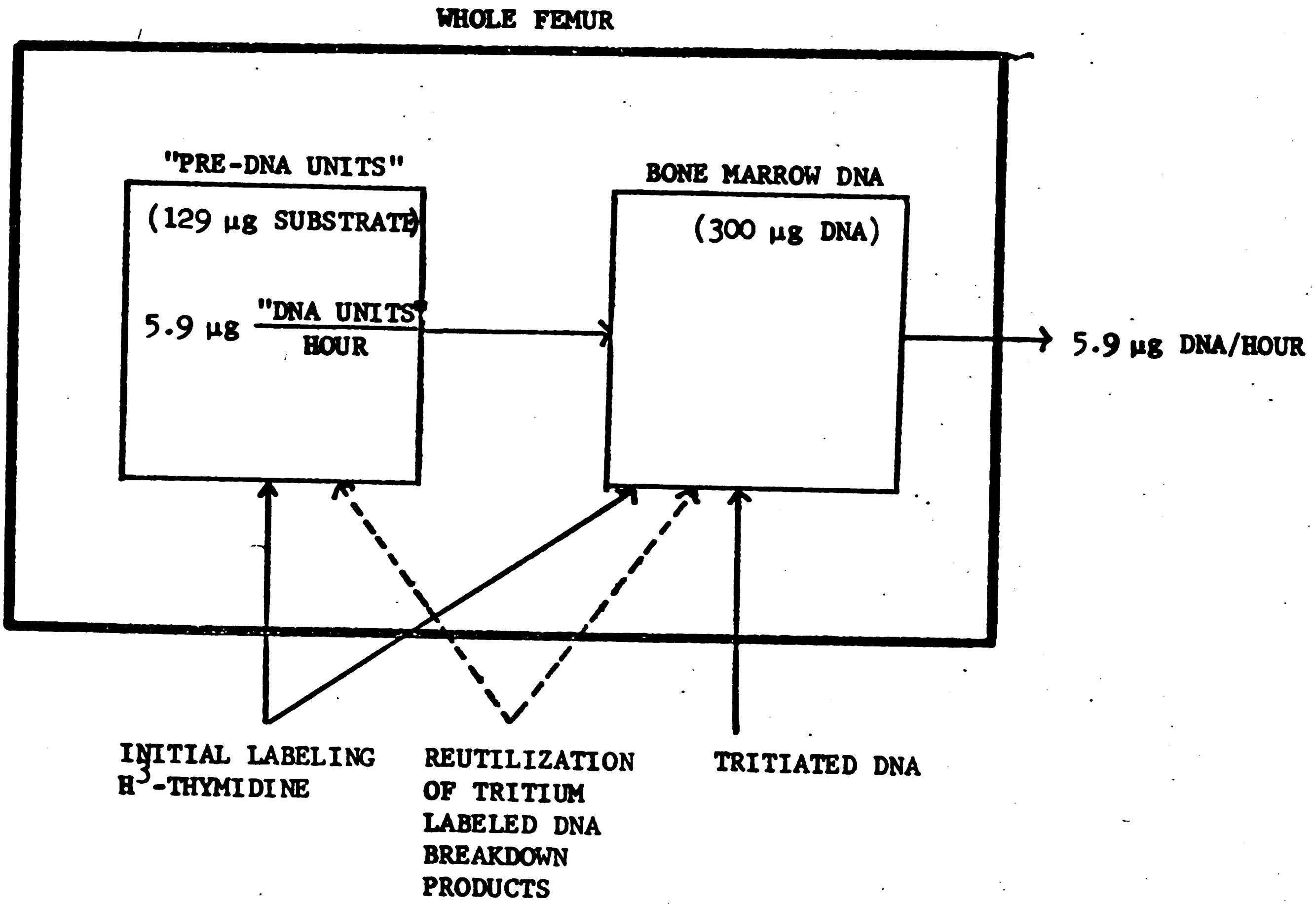
*Units giving rise to 129 micrograms of DNA.

FIGURE 13. A MODEL FOR THE MOVEMENT OF TRITIUM LABEL IN THE FEMUR BONE MARROW FOLLOWING A SINGLE INJECTION OF H^3-THYMIDINE.



Evaluation of Change in Activity in Bone Marrow Deoxyribonucleic Acid Between 18 and 24 Hours

The increase in activity in the marrow between 18 and 24 hours is of considerable interest. A similar rise in DNA activity has been reported for gut and spleen DNA (deLesdain and Pacletti, 1962). Experiments on reutilization of tritium activity in demand situations (i.e., tumor growth and liver regeneration) performed by Bryant (1962) and Rieke (1962) suggest the lymphocyte as a carrier of DNA for the synthesis of new precursors for DNA. However, Osmond and Everett's data (1964) show that only a few labeled cells (1/100) appear in marrow of a femur that has had its blood supply occluded during the availability period of H^3-thymidine. Bond et al. (1964) describe similar results in an experiment using rats in which labeled cells are collected from a donor and transfused into an unlabeled recipient. Evidently, there is no accumulation of labeled cells in the marrow, and the few labeled cells found represent the normal flux of leukocytes into the tissue from the circulating blood. Therefore, even though the mean grain count per cell is higher in lymphocytes than in the granulocytes in the peripheral blood (Figures 10 and 11) it is unlikely that this difference is responsible for the rise in activity at 18 to 24 hours. However, the implication is that there is preformed DNA activity entering the femur bone marrow DNA activity from outside the femur since the femur DNA pool size does not change. In addition, this activity is entering only the DNA pool and not the pre-DNA pool. This is so because the activity in DNA and femur are not significantly different at 24 hours.

Estimation of the Relative Contributions of Granuloid, Erythroid and Lymphoid Cells to the Deoxyribonucleic Acid Turnover in the Normal Mouse

It is of interest to consider the differences in normal and hypertransfused mice in more detail. It was pointed out that the DNA output in the hypertransfused mouse was 2/3 of the output in the normal mouse (Figures 7 and 9). Since the relative granulocyte distribution count in the hypertransfused mouse has been increased by a factor of two, the granulocyte output in the hypertransfused mouse must be about twice the output of the normal animal (Table I). From these data, it is possible to write two simultaneous equations in three unknowns.

For the Normal Animal: $R + G + L = 5.9$ micrograms/hour/femur (Pool size x rate constant = output or 300 micrograms DNA/femur x 0.019/hour = 5.9 micrograms/hour/femur)

For the Hypertransfused Animal: $2G + L = 3.06$ micrograms DNA/hour/femur (300 micrograms DNA/femur x 0.01021/hour = 3.06 micrograms DNA/hour/femur)

Where R = Red Cell DNA Output
G = Granulocyte DNA Output
L = Lymphocyte DNA Output.

These equations can be put in terms of the DNA output for red cells and the DNA output for granulocytes.

$$R = 4.35 - 1/2\ L$$

$$G = 1.5 - 1/2\ L$$

If one takes Osmond and Everett's figure (1964) for the average time spent by the lymphocyte in the bone marrow of 72 hours, then the rate constant for lymphocyte DNA output would be 0.014/hour. Since the total DNA pool in the marrow due to the lymphocyte is about 75 micrograms/femur, the turnover of lymphocyte DNA can be calculated to be 1 microgram/hour. If this value is substituted into the above equation, the red cell DNA output becomes 3.85 micrograms/hour/femur, and the granulocyte DNA output 1.05 micrograms/hour/femur. The figure presented earlier in the Discussion for red cell production (0.55×10^6 cells/hour/femur) can be put in terms of DNA output using 8.9×10^{-6} microgram DNA/cell -- See Appendix V. This value then becomes 4.9 micrograms DNA/hour/femur. It is evident that the erythroid population is the largest contributor to the total DNA turnover on the basis of these data.

One can make this estimate for the rat using Steels' data (rate constant 0.02/hour) and Donohue's figure for the total nucleated cell count in the rat (17×10^9 cells/kilogram) (Donohue _et al_., 1958). If there are 8.9×10^{-6} microgram DNA/cell, the DNA output due to the total bone marrow is 3000 micrograms DNA/hour/kilogram. The red cell production rate is 290×10^6 cells/hour/kilogram (Patt, 1957), therefore, DNA output due to red cells would be 2500 micrograms DNA/hour/kilogram.

This arithmetic gives only a rough approximation of DNA turnover of the individual components that make up the bone marrow. The turnover of each component in relation to the other in this instance has been made on the basis of its cellular distribution. Therefore, specific information on cell loss or recycling cannot be calculated from these data.

If one knew the activity incorporated into each cell type an evaluation of its turnover could be made. It would be possible to do this if one had autoradiographs of the bone marrow. A quantitative evaluation of the relative amount of thymidine incorporated into each component could then be made.

SUMMARY AND CONCLUSIONS

A comparison between the uptake of H^3-thymidine into mouse femur bone marrow DNA and into whole mouse femur has been made. In addition, certain other physiological effects were observed with the following observations and conclusions.

1. H^3-thymidine and its breakdown products are distributed throughout a volume as large as the total body water in not more than three minutes. H^3-thymidine and its breakdown products are cleared from the plasma very rapidly and with a time course described by a power function rather than by a simple exponential function. The maximum initial uptake from the plasma is attained in 1 hour in both the whole femur and in femur bone marrow DNA. By 1 hour, the plasma activity is much lower than either the whole femur or the femur bone marrow DNA activity. After the maximum uptake is reached, the activity in femur bone marrow DNA remains constant through 18 hours, while that in the whole femur begins to drop. The rate of loss from the whole femur is much slower than the rate of loss from the plasma.

2. An examination of hypertransfused mouse whole femur activity shows a slower rate of loss of activity after 24 hours than is observed in the normal femur. A first approximation shows that by far the greatest contribution to DNA turnover in the normal mouse femur bone marrow is made by erythroid DNA turnover.

3. The amount of DNA per femur was determined to be 300 micrograms per femur (3.4×10^7 cells per femur).

4. A comparison between this work and other published data shows that with the same total dose (0.5 microcuries per gram body weight), the percentage uptake of activity by the femur is independent of the specific activity injected, within the range of 1.9 to 6.0 curies/millimole thymidine. A large endogenous pool must be present, which dilutes the activity so that a tracer experiment is obtained over a wide range of specific activities.

5. The activity in femur bone marrow DNA remains constant for 18 hours, then between 18 and 24 hours increases to at least twice its pre-18-hour value. After 24 hours, a decrease in femur bone marrow DNA activity is observed, whose rate is not significantly different from the loss of whole femur activity during the first 18 hours.

6. The whole femur loses activity for the first 18 hours, then between 18 and 24 hours a rise of about 1.3 times the 18-hour value is observed. The amount of activity in the whole femur decreases at about the same rate as the pre-18-hour value. These rates are not significantly different from the rate of loss in activity observed in the femur bone marrow DNA after 24 hours.

7. The differences between the activity incorporated into DNA and its change over the first 18 hours, and the whole femur and its change in activity over the first 18 hours are resolved by postulating a labeled substrate pool for DNA. This pre-DNA pool

is labeled at the time of the initial injection. The same amount of activity enters the DNA substrate pool as entered the cells which are in DNA synthesis. The labeled substrate pool is of sufficient size such that it feeds label into DNA for about 18 hours.

8. The activity which enters both femur bone marrow DNA and the whole femur between 18 and 24 hours is in DNA *per se*, and not in the labeled substrate or precursor pool mentioned above.

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Appendix I

Coulter Electronic Cell Counter Standardization for Nucleated Mouse Bone Marrow Cells

Table III presents data from four experiments comparing electronic cell counts at various dilutions with a hemocytometer count. Bone marrow cells were collected from a mouse femur. Aliquots of these cells were taken and various dilutions made for the electronic cell count. A 0.5 percent Saponin Solution was used to lyse red blood cells. A hemocytometer count was made using a 1:20 dilution in 4 percent acetic acid. These results show that the Coulter counter is reliable over a fairly wide range of dilutions and that the results obtained compare well with the observed cell counts on a hemocytometer, at the usual dilution of 1:500. Richar and Breakell (1959) show that the standard error involved in a hemocytometer count is 20.55 percent as compared to 7.56 percent for the Coulter counter and Coulter (1956) and Mattern, Brackett and Olson (1957) show that the most accurate counting range is from 20,000 to 80,000 counts/0.5 milliliter. Since a 1:500 dilution of the samples gave 20,000 counts/0.5 milliliter, this dilution was chosen even though the least deviation for the hemocytometer could appear to be 1:1000, or slightly greater.

The data shown in Figure 14 are paired counts from the Coulter counter. The counts obtained are reproducible over the range studied.

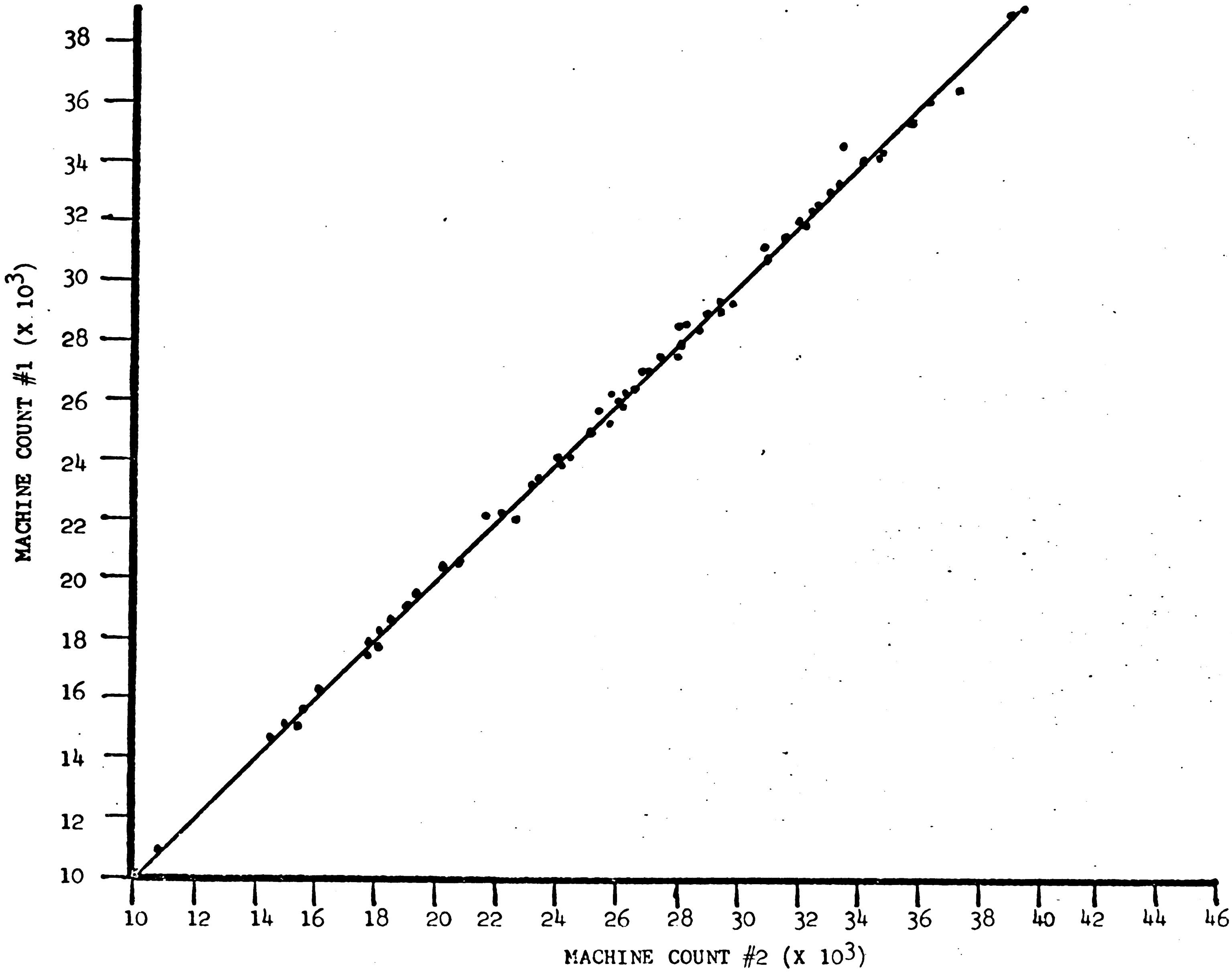

FIGURE 14. PAIRED COULTER COUNTS COVERING THE RANGE USED IN THESE EXPERIMENTS.

TABLE III

COMPARISON OF HEMOCYTOMETER COUNT TO COULTER ELECTRONIC CELL COUNTER COUNT AT VARIOUS DILUTIONS

Experiment I Hemocytometer Count -- Total Cells = 11.5×10^{6}(1:20 dilution)

	Machine	Corrected Machine Count	
Dilution	Cell Count	Total Cells	% Error
1:10,000	1.7×10^{3}	17.6×10^{6}	+ 53.0
1: 5,000	2.8×10^{3}	13.6×10^{6}	+ 18.3
1: 4,000	3.2×10^{3}	12.3×10^{6}	+ 7.0
1: 2,000	6.9×10^{3}	11.8×10^{6}	+ 2.6
1: 1,000	12.8×10^{3}	11.2×10^{6}	- 2.6
1: 500	21.8×10^{3}	10.9×10^{6}	- 5.2
1: 400	27.8×10^{3}	10.1×10^{6}	- 12.2
1: 200	55.4×10^{3}	11.0×10^{6}	- 4.3

Experiment II Hemocytometer Count -- Total Cells = 17.8×10^{6}(1:20 dilution)

	Machine	Corrected Machine Count	
Dilution	Cell Count	Total Cells	% Error
1:10,000	2.7×10^{3}	27.5×10^{6}	+ 54.5
1: 5,000	4.6×10^{3}	18.5×10^{6}	+ 3.9
1: 2,000	9.4×10^{3}	18.8×10^{6}	+ 5.6
1: 1,000	17.3×10^{3}	17.4×10^{6}	- 2.2
1: 400	42.0×10^{3}	16.8×10^{6}	- 5.6
1: 200	80.6×10^{3}	16.1×10^{6}	- 9.6

Experiment III Hemocytometer Count -- Total Cells = 8.8×10^{6}(1:20 dilution)

	Machine	Corrected Machine Count	
Dilution	Cell Count	Total Cells	% Error
1: 1,000	9.1×10^{3}	9.1×10^{6}	+ 3.4
1: 500	17.0×10^{3}	8.5×10^{6}	- 3.4
1: 400	20.6×10^{3}	8.2×10^{6}	- 6.8
1: 200	39.2×10^{3}	7.8×10^{6}	- 11.4

TABLE III (continued)

Experiment IV Hemocytometer Count -- Total Cells = 18.0×10^6 (1:20 dilution)

Dilution	Machine Cell Count	Corrected Machine Count Total Cells	Corrected Machine Count % Error
1: 1,000	14.4×10^3	14.4×10^6	- 20.0
1: 500	28.6×10^3	14.3×10^6	- 20.6
1: 400	43.6×10^3	17.4×10^6	- 3.3
1: 200	86.0×10^3	17.2×10^6	- 4.4

Appendix II

Standardization of DNA Activity Technique

Twenty-eight mice were injected with 10 microcuries of H^3-thymidine via the tail vein. Approximately 15 hours later, bone marrow cells were removed from both femurs of each mouse. These cells were obtained and processed as indicated in the Materials and Methods section under DNA extraction. The cells were made up to a known volume, and twelve aliquots were made. Before the DNA extraction was begun, eight samples were spiked with DNA from the standard solution: four with 40 micrograms and four with 100 micrograms. In addition to the twelve samples, two 100 microgram aliquots from the DNA standard solution were processed through the extraction procedure. All samples were processed and counted on the TriCarb counter as indicated in the Materials and Methods under DNA counting. Table IV presents the results of this standardization.

TABLE IV

STANDARDIZATION OF DNA ACTIVITY TECHNIQUES

Sample	Amount of DNA added	µc DNA extracted	µg DNA extracted minus µg DNA added	Count/minute	Count/minute
1	0	220	220	2067	9.40
2	0	230	230	2128	9.25
3	0	240	240	2217	9.24
4	0	230	230	2038	8.86
5	40	277	237	2099	8.86
6	40	280	240	2264	9.43
7	40	279	239	2325	9.73
8	40	262	222	2165	9.75
9	100	319	219	1973	9.00
10	100	335	235	2232	9.50
11	100	315	215	2079	9.67
12	100	320	220	2115	9.61
Std. 1	100	99	---	Background	---
Std. 2	100	90	---	Background	---
N = 12			$\bar{x}$ = 229 µg DNA S.E.M. = ± 2.7 µg DNA	$\bar{x}$ = 2142 counts/minute S.E.M. = ± 29.3 counts/minute	$\bar{x}$ = 9.36 count/minute/µg DNA S.E.M. = 0.276 count/minute/µg DNA

Where:

$\underline{N}$ = sample size

$\bar{x}$ = mean = $\frac{\underline{EX}}{N}$

S.E.M. = standard error mean = $\sqrt{\frac{E(x - \bar{x})^2}{N(N - 1)}}$

Appendix III

Standard Curve - DNA

A sample of highly polymerized pure deoxyribonucleic acid was obtained from Nutritional Biochemicals. A stock solution was prepared by dissolving DNA in 5 millimoles sodium hydroxide (Burton's Technique). A standard curve obtained from such a preparation is shown in Figure 15. New standard curves were run and tested for uniformity frequently. In addition, an aliquot of the stock solution was run with each experiment.

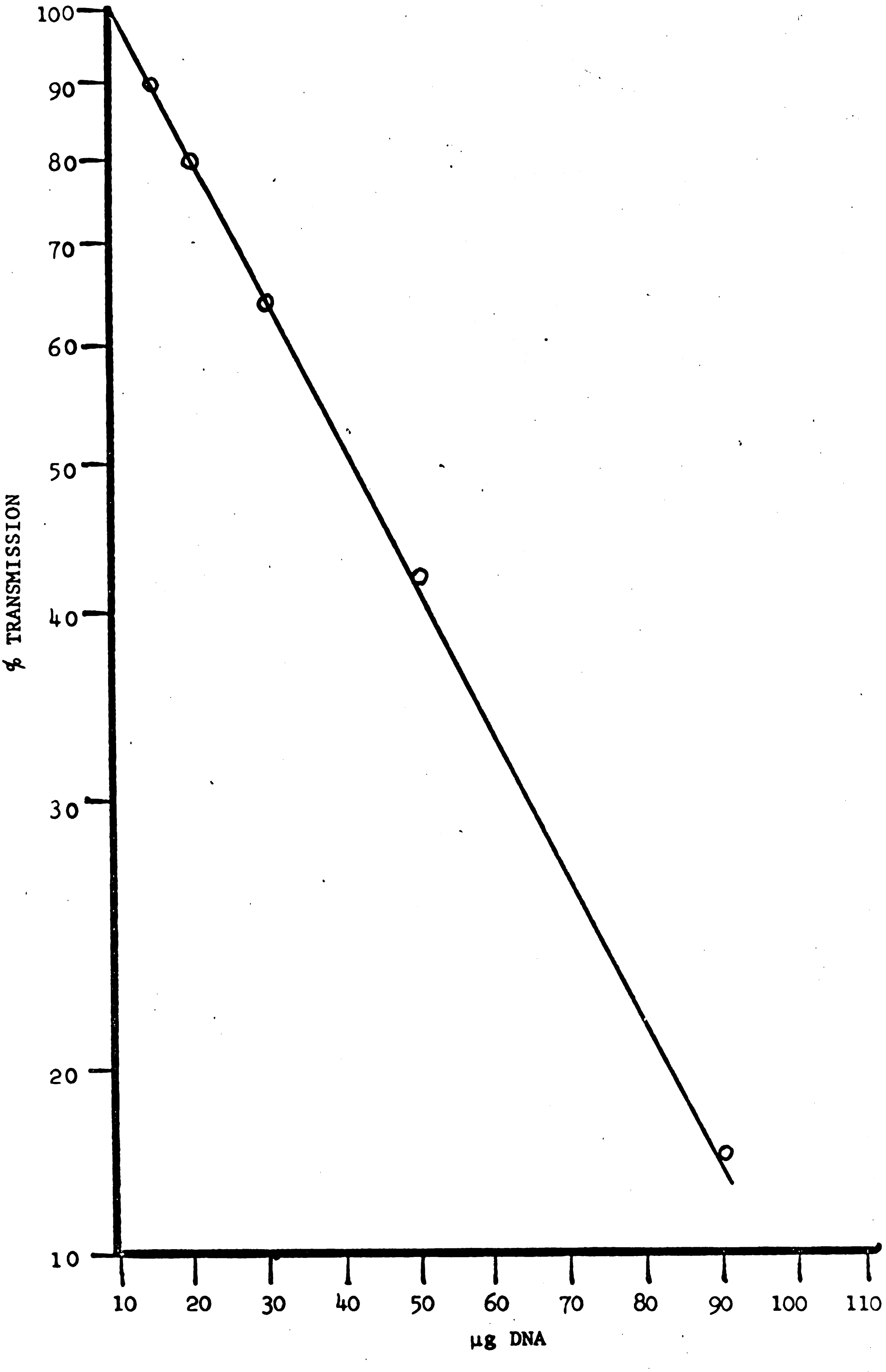

FIGURE 15. STANDARD CURVE FOR DNA.

Appendix IV

Standardization of Femur Combustion Technique

Twelve mice were sacrificed and both femurs removed as indicated in the Materials and Methods section. These femurs were dried at 90° Centigrade for two days, then two femurs were wrapped in black paper on which 0.09 microcurie of H^3-thymidine was dried. Twelve combustions were carried out as described in the Materials and Methods. The mean of these combustions was 0.091 microcurie with a standard error of mean of ± 0.0006. (For the equations used to calculate these statistics see Table IV).

Appendix V

DNA Content Per Cell

The DNA content per cell was measured in all experiments involving DNA extraction. Figure 16 presents data showing that the DNA content per cell remains constant over the range of values in these experiments. (Intercept = 40.96 ± 11.02; Slope = 7.42 ± 0.357). When the amount of DNA per cell is pooled (i.e., data from Figure 16), the mean is 8.9×10^{-6} microgram DNA/cell with a standard error of the mean of $\pm 0.084 \times 10^{-6}$. (For the equations used to calculate these statistics see Table IV).

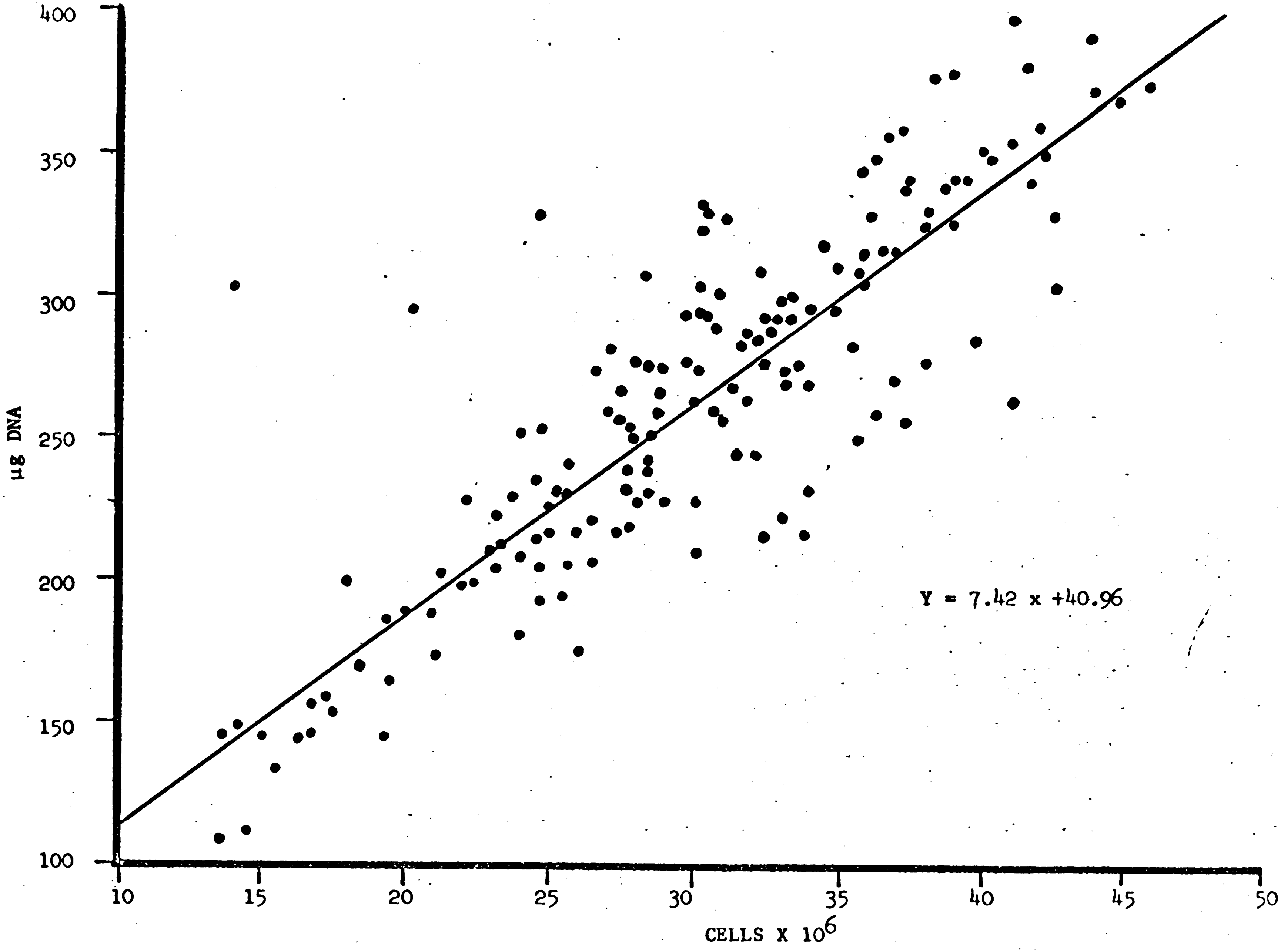

FIGURE 16. AMOUNT OF DNA EXTRACTED AS A FUNCTION OF THE CELL NUMBER.

Appendix VI

Basic Data

The three tables (V, VI and VII) that make up this appendix are the basic data in the thesis.

TABLE V

RESULTS OF INDIVIDUAL EXPERIMENTS USING DNA EXTRACTION IN NORMAL ANIMALS

Time	Experiment Number--All Values $\mu c/\mu g$ DNA X 10^{-6}							
(Hours)	1	2	3	4	4'	5	11	15
0.5				68.6				
1		70.0	61.6	77.8				60.2
2		74.0	81.0	75.2				64.2
4		90.2	68.8	78.8	74.6	70.6		50.0
6	101.2	85.8	63.0	65.2		65.0		55.0
8				59.2		86.8		80.8
10				76.2		84.6		
12	75.8	95.4		53.0		66.4		54.8
16								102.0
18	60.6	90.2				66.4		82.0
24		75.6					121.6	154.2
30		77.6						
36		108.8						
42							95.4	
48		95.6						
60		85.6					39.4	
72		71.2					34.8	
84							39.4	
96							50.4	
108							19.8	
120							15.8	
132							8.2	
144							8.4	
168							10.8	

TABLE VI

RESULTS OF INDIVIDUAL EXPERIMENTS USING FEMUR COMBUSTION IN NORMAL ANIMALS

Time (Hours)	Experiment Number--All Values μc/Femur									
	3	4	5	6	7	8	9	10	11	12
0.25				0.0337						
0.5		0.0440		0.0346						
1	0.0460	0.0510		0.0374		0.0477				
2	0.0475	0.0420		0.0468			0.0331		0.0494	
4	0.0435	0.0424	0.0427	0.0462	0.0456	0.0405	0.0500			
6	0.0430	0.0400	0.0444				0.0343			
7				0.0415						
8		0.0350	0.0464			0.0327	0.0329			
10		0.0384	0.0411				0.0364			
11				0.0360						
12		0.0300	0.0342			0.0339			0.0352	
14				0.0357		0.0344	0.0361			
16				0.0330		0.0352				
18			0.0271		0.0426	0.0312	0.0329		0.0315	
20							0.0298			

TABLE VI (continued)

Time	Experiment Number--All Values µc/Femur									
(Hours)	3	4	5	6	7	8	9	10	11	12
22							0.0271			
24							0.0315	0.0338	0.0564	
26								0.0415		
28								0.0396		
30								0.0384		
32								0.0331		
36										
42									0.0366	
48										0.0213
60									0.0253	
72									0.0256	
84									0.0156	0.0139
96									0.0202	
108									0.0120	0.0066
120									0.0084	
132									0.0076	0.0064

TABLE VI (continued)

Time (Hours)	Experiment Number--All Values µc/Femur 3	4	5	6	7	8	9	10	11	12
144									0.0065	
168									0.0063	0.0039

TABLE VII

RESULT OF INDIVIDUAL EXPERIMENTS USING FEMUR COMBUSTION IN HYPERTRANSFUSED ANIMALS

Time (Hours)	Experiment Number--All Values μc/Femur 8	9	10	12
1	0.0463			
2		0.0463		
4	0.0411	0.0500		
6		0.0433		
8	0.0371	0.0345		
10		0.0393		
12	0.0370			
14	0.0331	0.0341		
16	0.0300			
18	0.0406	0.0391		
20		0.0273		
22		0.0219		
24		0.0301	0.0351	
26			0.0460	
28			0.0351	
30			0.0465	
32			0.0416	
48				0.0300
84				0.0219
108				0.0179
132				0.0114
168				0.0092