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ENIGMAS UNDERLYING THE STUDY OF HEMOPOIETIC CELL PROLIFERATION.*

By

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DISCLAIMER

A discussion of enigmas amidst the beautiful exposition of scientific accomplishments presented at the Symposium in honor of Dr. Donald Dexter Van Slyke is a conundrum in itself. This Symposium held on the occasion of his 80th anniversary was an outstanding success in terms of scientific excellence and is a great tribute to the continuing intellectual, scientific and physical vigor of Van Slyke.

A very wide spectrum of scientific disciplines were covered to wit, the relation of chemical structure to function, the exquisite elucidation of vitamin B_{12} structure by x-ray diffraction and analysis, structure of less well defined chemical entities such as collagen, collagen function, ion transport, biochemical changes in cholera, renal changes in shock, immunity in collagen disease, hypertension and lipid metabolism. Most of the studies presented, concerned truly major advances in knowledge, made possible by the development of new techniques, of new methods, or of interpreting old techniques. The Symposium in large part was concerned with organs (intestine, kidney, adipose tissue, lymphoreticular tissue, connective tissue etc.). However, cells as cells, and tissues as tissues were given but scant consideration in the classical anatomical or cytological sense. This is not surprising; biological science started as a morphological description (Phylla, Classes etc., Gross Anatomy). The textbooks for these have long since been written. There is very little to add in the classic concept. At the molecular level precise knowledge on structure, function and information coding is accumulating at a dizzy pace. These are truly exciting times in biology. However, there remains a hiatus in accumulation of knowledge between the gross anatomical

structure of animals and tissues and their function on one side and the molecular structure and function of biologically active molecules on the other side. In between lie the cells as highly structured units with active interphases, intracellular concentrations of specific chemical entities in organelles with their own ultrastructure, intracellular and extracellular communication network. Cells are certainly not and can not be treated as dilute solutions of enzymes and substrates.

Knowledge on the ultrastructure of cells, chemical nature of the ultrastructure, structure of complex molecules grows rapidly with improved chemical techniques. The resolution of the electron microscope is approaching molecular dimensions and combined with enzymatic degradation etc. may well demonstrate precise structure. Despite the rapid growth of knowledge alluded to above and presented so eloquently at this Symposium, there remains a particular dearth of knowledge in respect to the chemical or other regulation of the "steady state" proliferation, differentiation and maturation of the cell renewal systems in mammals. Conceptual advances in this area were dealt a serious blow with the recent death of Henry Quastler who had contributed so much to the development of methods and knowledge on cell proliferation and its specific study in the gastrointestinal tract (60-63).

Accordingly, as a hematologist, I would like to address myself to certain facets of blood cell production and its regulation. Needless to say, but often neglected in the search for biochemical control mechanisms of diverse sorts, is the simple fact that death of the animal will follow cessation of production of new red cells, platelets, granulocytes or lymphocytes. As a physician, who all too frequently has observed death result from spontaneous, drug or radiation induced failure of new cell production, I have been drawn to speculate and to experiment on the regulation

of production and thus welcome this opportunity afforded members of the Van Slyke Symposium Organizing Committee to indulge in some speculation and to recount some experimental approaches to hemopoietic cell regulation. Thus I am honored and pleased to be able to contribute to the Van Slyke Festschrift.

Steady State Catenated Proliferating Cellular Systems:

All hemopoiesis can be described as consisting of a series of cytologic compartments in which proliferation and maturation continue in parallel. This is shown schematically in Figure 1 without reference to any particular cell line. The generally accepted concept demands an undifferentiated stem cell pool. Cells in this pool are believed to be acted upon by a specific inductor (generally not identified) which induces a loss of cells from this pool by differentiation into a specific cell line. From this point onwards, the cells become identified as belonging to a specific cytologic line. Thereafter these cells go through a series of proliferating mitoses and maturation simultaneously. After a certain degree of maturity is attained, mitosis ceases and maturation continues in the non-proliferating pool. At a later stage, the cells attain a degree of maturity (chemical maturation) that permits entrance into the functional pool. At a later stage, cell loss occurs as a result of senescence, functional destruction or loss from the functional compartment. Since there are clearly four or more cell systems which go through a sequence as described above maintaining essentially a "steady state equilibrium" there must either be feed back loops from the losses in the functional pool to the stem cell and perhaps also the intermediate stages in order to regulate the system and adapt to unexpected increases in demand or perhaps the whole system is programmed genetically.* Before proceeding to a discussion of erythropoiesis, granulopoiesis and lymphopoiesis, a closer look at the stem cell concept is indicated.

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* See Quastler, H. (60) for problems concerned with feed back control, cybernetics, programmed systems etc. in relation to cell proliferation.

Stem Cell Concept:

The stem cell concept is many decades of age and is described in detail by Bloom (6). It is difficult to trace the entire history with satisfaction. Cowdry (14), Bloom (6) recognized the need for a self perpetuating cell. Whether there is a single stem cell or multiple stem cells is as yet equivocal. In embryonic development, blood formation is commenced by the primitive mesenchyme in the yolk sac. A vast literature primarily of polemics exists as to whether there is a single stem cell for hemopoiesis or multiple cells in adult life (reviewed in Downey by Bloom) (6). The number of stem cells in adult life remains still unknown. The existence of pure aplasias of one type or another in human disease can be used as an argument for the loss of the stem cell for that line or the loss of the specific inductor for that cell line which impinges upon a common stem cell. Radiation protection studies reviewed by Bond et al (7) and Goodman et al (30) clearly show that the common stem cell(s) passes through the blood. The British group, Barnes, Loutit and Ford (4) clearly have shown that the only source for all the stem cell(s) is the bone marrow. What is more distressing is the failure so far to satisfactorily identify the stem cell(s) cytologically. Fliedner et al (26) and Cudkowicz et al (23) have identified tentatively the stem cell as being respectively a small dense mononuclear cell in the canine bone marrow and a small, mononucleated lymphocyte like marrow cell in the mouse not derived from lymph nodes. Fliedner et al (26) "conclude that the 'stem cell(s)' responsible for the repopulation of the marrow must be mononuclear cells which are in a quiescent state or have a long generation time under normal steady state conditions so that under these conditions they rarely are found in DNA synthesis and thus are not labeled". There seems to be little direct support today for Yoffey's

ideas (70) tha the small blood lymphocyte migrates to the bone marrow and acts as a stem cell for erythropoiesis although this possibility has not yet been disproved. Since lymphocytes do migrate in some degree to the marrow (41,70) it is conceivable that they may slowly become "activated" into stem cells by the marrow environment in some undisclosed manner. Cudkowicz (23) most recent work on separation of cells in the mouse marrow by glass wool filtration strongly supports the idea that the mouse stem cell is a small marrow lymphocyte which protects against radiation injury whereas lymph node lymphocytes with the same appearance do not protect although it remains conceivable that anatomic residence within the marrow parenchyma imparts myelopoietic stem cell properties to lymphocyte immigrants within the marrow. For the sake of our discussion one must confess that the absolute identity of a common stem cell remains an enigma. However, the stem cell concept is necessary to make observations on hemopoietic cell proliferation rational. If the most immature member of each cell line acts as its own stem cell there is still the factual dilemna that these cells are not seen in concentrates of the peripheral blood yet the evidence for the existence of radioprotection by peripheral blood cells repopulating all hemopoietic tissues remains on solid grounds (8,30).

Osgood (55) was the first to question whether the rigid asymmetric mitosis (one daughter stem cell is differentiated the other remains as a stem cell) would maintain a normal steady state production and also respond to increased rates of production or permit regeneration. He proposed a system in which stem cells could divide either exponentially to expand the stem cell population (hisα - 2α mitosis) or divide asymmetrically (hisα - α n mitosis). Lajtha et al (46, 47 discusses the stem cell problem and proposes a model that is presumed

to be able to respond flexibly to diverse depletions such as radiation injury. His scheme for the stem cell compartment is based on an analogy to liver regeneration after partial hepatectomy. He assumes in the normal steady state that only a small random fraction is proliferating symmetrically* to meet the demands of replacement from senescence and small random losses. The quiescent fraction is subject to differentiation by specific inductors such as erythropoietin when an increased need develops from peripheral loss of Rbc's. When the quiescent stem cell fraction decreases in size it must be rebuilt by an increased production of new stem cells. Obviously there is a complex system of sensors and signals operating to prevent undue depletion of the stem cell pool and unduly wide fluctuations in total size of the stem cell pool as it responds. Lajtha's scheme has been programmed and tested for its theoretical capability of responding to needs, radiation injury etc. and has been shown in principle on computer analysis to be a responsive and self-damping system that does not permit wild fluctuations. Furthermore, his model is consistent with some experimental observations (Lamerton et al (48) and Gurney et al (35).

His idea of a large quiescent fraction is supported experimentally by the observations of Fliedner et al (26) and Cudkowicz et al (23) upon human,

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* Although Lajtha finds the idea of the asymmetric division unacceptable for hemopoiesis there are clear cut examples of asymmetric mitosis eg. grasshopper neuroblast by Carlson plant meristems, animal eggs with spiral cleavage etc. see Mazia (53).

canine and mouse bone marrow. Furthermore in another tissue, the periosteum, Tonna and Cronkite (68) have shown that there is a large quiescent population of pre-osteoblasts and osteoblasts that await a signal for proliferation such as fracture. Thus the idea of quiescent potentially active cells is compatible with some experimental observations.

The stem cell pool has been visualized by Johnson (40) as a cytologic compartment with autonomous proliferation in which the population doubles with each generation so that

$$N = N_o \cdot 2^{\frac{t}{t_G}} = N_o \cdot (.693)^{\frac{t}{t_G}}$$

and the growth constant $\lambda = \frac{.693}{t_G}$ or $N = N_o e^{\lambda t}$ where

t_G = generation time

t = time from t_o to t, $\frac{t}{t_G}$ the number of generations (n).

$\frac{dN}{dt} = \lambda N$ the growth rate in an expanding population.

If rate of removal from the expanding population is $\lambda_1 N$ from death or differentiation then the net rate of change is

$$\frac{dN}{dt} = \lambda N - \lambda_1 N$$

and in the steady state is zero.

If the preceding model is true then one can visualize considerable flexibility providing the stem cell pool is "size conscious". Following an unexpected drain of cells one could imagine the remainder will not be susceptible to differentiation until the size is restored. If there is a sizeable death function normally there is immediately available for differentiation the fraction ordained to death in the normal "steady state". Furthermore

the temporary restriction of no differentiation no death with the same generation time will permit the stem cell pool to rebound exceedingly rapidly. If one invokes a system in which a shortening of the generation time is feasible there will be an exceptionally rapid response.

One can construct models ad infinitum and imagine means by which the model will explain biologic phenomena. However, this is purely an intellectual exercise unless one can verify the model and its operation by experiment. For other discussions of the stem cell see Johnson (40), Osgood (55) and Lajtha et al (35, 46,47).

Proliferating Pool of Differentiated Cells:

The main characteristic that is imparted to a differentiated pool is multiplication but inability for self prepetuation. If the input fails, the compartment vanishes as maturation continues. Such a compartment is diagrammed in Figure 2. The compartment is defined in terms of cytoplasmic and nuclear structure. Cells may divide within the cytologic compartment or may be in transit through it while maturing. Since mitosis is seen some cells do divide. Since "flash labeling" by tritiated thymidine is observed some cells spend at least some of the time within the cytologic phase in DNA synthesis. There is no way to determine if all cells that enter a cytologic phase divide within this phase one or more times or if they divide at all. However, it is tacitly assumed by some (46, 47, 58, 59) that one or more mitoses occur at several cytologic phases. Conceptual and mathematical problem of compartment transit time, and intracompartmental growth have been considered in detail by Johnson (40), Killmann et al (43) and Patt (58).

The following are clear for any compartment between the stem cell and

the first non-proliferating compartment.

$$K_{out} = K_{in} + \frac{N_M}{t_M} \qquad t_M = \text{mitotic time} \qquad \text{Equation (2)}$$

$$K_{out} = K_{in} + \frac{N_S}{t_S} \qquad t_S = \text{DNA synthesis time} \qquad \text{Equation (3)}$$

where K_{out} equals the rate of leaving the compartment, K_{in} equals the rate of entry, N_M equals number in mitosis, N_S equals the number in DNA synthesis. $\frac{N_M}{t_M}$ and $\frac{N_S}{t_S}$ equals the intracompartmental growth rate.

The entire proliferating pool as shown in Figure 1 consists of a series of cytologic phases. If the stem cell input K_{in} is known then the efflux (K_{out}) from the terminal dividing cytologic phase can be expressed as follows (10,25):

$$K_{out} = 2^n \cdot K_{in} = 2^{\frac{t}{t_G}} \cdot K_{in} \qquad \text{Equation (4)}$$

where t equals the sum of the cytologic phase transit times (40) and t_G equals generation time and n equals the average number of mitoses or $\frac{t}{t_G}$. These simple principles will be applied later in the case of erythropoiesis and granulopoiesis.

METHODS OF STUDY

Precise relatively fast methods of labeling DNA with phosphate, purine and pyrimidine precursors are available after which one can follow the specific activity of DNA providing one can separate specific cell lines. The later has not been accomplished in the case of the bone marrow or lymph nodes. When it has been attained (separation of blood granulocyte by Ottesen (56) one must resort to mathematical interpretations of DNA specific activity curves of a

very complex nature.* DNA specific activity interpretation will not be discussed here because the major concern is with interpretation of H^3TDR autoradiographic data. However, the relative simplicity in interpretation of labeled red cell data (N^{15} glycine) where the labeled cohort enters the blood over a short period, remains constant for a long period and disappears over a short period by the mathematical equations which Shemin and Rittenberg (64) used beguiled many hematologists into applying the same equations to leukocyte DNA specific activity curves. Cornfield (10) summarized the mathematical complexity of the leukocyte problem and eloquently posed the following. "The moral for the 19th century hematologist, then, is that if one is interested in even so elementary a measurement as average sojourn,** it takes a certain amount of nontrivial mathematical analysis to establish whether the conditions for its estimation exist."

The obvious answer to the dilemna was direct DNA labeling followed by a cytologic and autoradiographic study so that one could follow the transit from one compartment to another of labeled cells. This became possible with the demonstration by Hughes et al (37) that tritiated thymidine was a satisfactory label for mammalian proliferating cells. The methods of getting information by the combined autoradiographic and cytologic study have been discussed in detail by Cronkite et al, (16-20) Quastler et al, (60-63) Johnson, (40) Bond et al (7) and Koburg (45). The problems of interpretation of data have been considered by Quastler (60) and Johnson (40) and need

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* For the interpretation of DNA specific activity curves see Stohlman, Kinetics of Cellular Proliferation, Chapter VI, Grune and Stratton, New York, 1959.

** For example, average time of granulocytes in blood from DNA labeling in marrow.

not be elaborated upon. The specific observations that have or can be made are:

a. Appearance of label in a non-proliferating compartment from a proliferating compartment e.g. myelocyte to the metamyelocyte.

b. Transit of labeled cells from a storage compartment to a functional compartment e.g. labeled granulocytes from marrow to the peripheral blood, from blood to an extravascular area (e.g. saliva).

c. Diminution in mean grain count of cells in a proliferating compartment or changes in distribution of grain count.

d. Index of labeling of a specific cell line and its subsequent changes.

e. The appearance of labeled cells in the "small mitotic window".

ERYTHROPOIESIS:

Histologists have long know that red cells are produced within the marrow because of the presence of mitosis in cells that contain hemoglobin. The capability of producing large numbers of red cells upon demand following hemorrhage, induction of hypoxia and hemolysis with a concomitant increase in mitosis within the marrow and reticulocytes in the blood is common knowledge. However, the existence of the "steady state" equilibrium with a turnover time for the erythrocytic mass awaited the determination of the life span of the red cell by Winifred Ashby (3) using a simple differential agglutination technique to distinguish the survival of transfused donor cells from the recipient's own cells. In these classical studies the life span of the human red cell was estimated as 120 days with cells being lost predominantly by simple senescence since random loss of red cells is almost negligible in the healthy state. Isotopic techniques (64) have clearly confirmed the life span to be 120 days with loss of cells by simple senescence.

Estimation of red cell mass and red cell survival by radioisotopic and dye dilution techniques have become commonplace and are routinely applied in psychologic states and under variously imposed exogenous stimuli. Some insight into duration of time from the earliest red cell precursors to emergence in the peripheral blood was gained by radioiron autoradiographic studies (1). However, it was by application of H^3TDR labeling of DNA and its subsequent movement by autoradiographic study that a finer time structure of erythropoiesis was gained.

In Figure 3 erythropoiesis is presented schematically. The various cytologic phases of erythropoiesis have been designated E_1 through E_7 to avoid cumbersome hematologic terms. However, for orientation E_1 represents the proerythroblast, E_4 the last dividing polychromatic normoblast, E_5 the non-dividing nucleated red cell precursor, E_6 the reticulocyte and E_7 the mature red cell.

In E_1 through E_4 mitosis and DNA labeling is observed hence at least a portion of the cells in these cytologic phases divide within that phase. It is conceivable also that labeling may take place in one cytologic phase and mitosis within a later cytologic phase. Some, perhaps all of the cells in transit through E_1 to E_4 pass through all of the cell cycle phases diagrammed in Figure 2. Mitotic index and H^3TDR data will be utilized to describe time and flow patterns.

MITOTIC INDEX:

The use of mitotic index for estimation of turnover and proliferation rates and the duration of some phases of cell proliferation in hemopoiesis has been considered in detail by Killmann et al (42-44) and in general by Hughes (36).

In Table 1, data from Killmann et al is abstracted and computations made

in respect to relative size of the erythrocytic marrow compartments, specific mitotic index (fraction of a cytologic compartment in mitosis), minimum and maximum compartment transit times. The principles underlying the computations are discussed in detail elsewhere (43). In making these computations, a mitotic time for E_4 of 0.58 hours was used since it is based on direct labeling observations of Odartchenko et al (54). Since Makino and Nakahara (57) showed mitotic time to increase with cell size, one might expect E_1, E_2, and E_3 to have a longer mitotic time. However, no adjustment was made in the absence of specific data. If one assumes that all E_4 cells divide and that this is a heteromorphogenic mitosis, one can then compute the generation time (t_G) of this cytologic class from the following relation between fraction in mitosis (I_M), number in mitosis (N_M), number in total population (N), mitotic time (t_M) and generation time (t_G)

$$I_M = \frac{N_M}{N} = \frac{t_M}{t_G} \qquad \text{Equation (5)}$$

Since one can not distinguish between E_1 and E_2 in mitosis, these are pooled into one class for this purpose.

In the calculation in Table 1, it is assumed that there is no stem cell input into E_1 and that there is no cell death in E_1 or E_2. It will be noted that there is a discrepancy between efflux from E_4 and influx in E_5. The latter is estimated from appearance of labeled DNA in the initially non-labeled E_5 of 5.3% per hour (Bond et al, 7). If one accepts these values one will conclude that there is a significant death at the E_4 level. Such has been proposed for granulocytopoiesis by Patt (58). However, as Bond pointed out the replacement rate may be significantly greater than that observed if labeled and unlabeled cells enter simultaneously. Furthermore, if one takes the estimate

of Killmann et al (44) or Donohue et al (24) for erythrocytic precursors in the marrow of man of about 5.0×10^9 nucleated red cell precursors per kg one can then estimate absolute cell production at the E_4 level as follows:

$$\frac{\text{Nucleated red cell precursors/kg} \times \text{fraction that is } E_4 \times \text{mitotic fraction}}{\text{mitotic time}}$$

$$\frac{5.0 \times 10^9 \times 0.31 \times 0.056}{0.58} = 15 \times 10^7 \text{ cells per hour}$$

At a concentration of 5.0×10^6 red cells per mm^3, 7% blood volume and 0.083% per day replacement the red cell requirements per hour per kilogram are:

$$\frac{70 \times 10^3 \times 5.0 \times 10^6 \times 0.0083}{24} = 12 \times 10^7 \text{ per hour}$$

hence it would appear that there is no significant loss at the E_4 level.

In Table 1, transit times of the various cytologic phases are tabulated from Killmann et al (44). The minimum time through E_3 and E_4 is 15.7 hours and the maximum is 67.7. Utilizing equation 4 and assuming a generation time of 9 hours applies to E_3 and E_4 one can compute the K_{in} from E_2 to have a maximum of 3.65×10^7 cells per hour per kg. and a minimum input of 6.59×10^5 per hour per kg. A mid point value appears to be more consistent with proportional distribution of cells in differential counts and likely generation time at the stem cell level.

Thymidine (H^3TDR) Labeling of Erythrocytic Precursors:

Erythropoiesis has been studied in vivo in man (Bond et al. 7) and (Cronkite et al,20) and in the dog (Odartchenko et al. 54, Cottier et al 8 and Bond et al. 9). In Table 2, results are compiled and some computations are made.

There are many blank spaces since it is not possible to determine the

finer time structure of the cytologic compartments when one feeds another. Satisfactory determinations are only made in the terminal initially labeled compartment E_4. Within this cytologic phase quite good estimates have been made in the dog (8,9,54). Unfortunately sufficient data are not available on human beings to check against the canine values but where comparable data are available, the dogs and man seem to be similar. In the estimations of proliferation rate the canine estimate of DNA synthesis time of seven hours for E_4 was used. There is a definite discrepancy between grain count halving time in man and generation time for E_4 in the dog. Since there are 1.55×10^9 E_4 cells/kg. in man and one needs 12×10^7 red cells produced per hour, one can check to see what half time is needed to produce sufficient new red cells from E_4 assuming that all E_4 divide and that there is no cell loss at this level. A generation time of about 12 hours is needed. This is fairly close to the observed canine generation time for E_4.

Since the grain count halving time is clearly incompatible with a generation time at E_4 of about 12 hours an explanation is needed. In a catenated series of compartments, the half time will become ultimately that of the slowest compartment. This would suggest that the compartment which feeds E_1 and E_2 has a generation time of 24 hours. However, this is incompatible with a labeling index of almost 1.0 which would necessitate a DNA synthesis time of almost 24 hours. Clearly something is amiss with grain count diminution and the explanation of the enigma is obscure. The difficulty may arise from technical problems with tritium autoradiography (geometry double hits on halide grains etc) or may result from reutilization of tritium within the marrow.

An explanation is recognition. If cytologic definition describes the appearance of a cell in DNA synthesis, the R_1 phase is lost. I_L approaches unity.

Another inconsistency is the labeling index in E_4 of 0.33. If t_S is truly about 7 hours and t_G about 9 hours, then one would expect a labeling index of 0.78 if each E_4 cell has a heteromorphogenic mitosis. Thus either the mitosis occurs early in this terminal cytologic compartment or many cells do not divide within the compartment. For the moment there is no clear explanation and thus another enigma is added to the currently unanswered problems.

GRANULOCYTOPOIESIS:

It is believed but not proved that there is a common stem cell for granulocytopoiesis. There is a proliferating maturing pool and a non-dividing maturing pool of cells as shown in Figure 1. There are a whole series of biochemical changes that proceed with maturation and are manifested by development of specific granules and cytochemically detectible enzymes. At the late myelocyte stage, cell cycling ceases and is followed by a series of characteristic morphologic changes in the nucleus. As the nucleus shrinks, specific granulation becomes more prominent and numerous cytologic enzymes increase in concentration and RNA synthesis decreases, the

cell becomes acceptable to the circulation and migrates through the marrow sinusoids into the blood. In apparent contrast to the red cell, where MCHC increase or RNA decrease seems to be correlated with cessation of cell cycling and nuclear extrusion there is no clear correlation between cessation of cell cycling and some cytoplasmic constituent(s). The flow of granulocytes, the relative production rates in the marrow, the release to and loss from the blood will be considered next.

In Figure 4, a panoramic view of granulocytopoiesis is presented. Production is clearly within the marrow with apparently a undirectional flow into the blood. Granulocytes leave the blood in two distinct manners- simple senescence and random loss into and through some epithelial surfaces. The lung is presented separately since all blood flows through the lung; the lung presents a huge epithelial surface vulnerable to bacterial infection; and it has been proposed that the lung has a specific regulatory function of granulocyte concentration in the blood (Ambrus, 2). Each of the preceding facets will be discussed.

Our data (7, 16-19) and that of Patt and Maloney (57-59) will be considered in respect to a model for granulocyte kinetics and particularly the flow pattern within the marrow.

The flow pattern for granulocytopoiesis is assumed to be stem cell $\longrightarrow$ myeloblast (M_1) $\longrightarrow$ promyelocyte (M_2) $\longrightarrow$ myelocyte (M_3) $\longrightarrow$ metamyelocyte (M_4) $\longrightarrow$ band (M_5) $\longrightarrow$ segmented neutrophile (M_6).

The stem cell proliferates autonomously and the total rate of differentiation equals proliferation rate, in order to maintain the steady state, myeloblast through myelocyte phases are compartments with proliferation. The total number of generations between these boundaries is not clear. In Table 3, the relative production rates from mitotic index data (42) are computed from principles laid down by Johnson (40), Killmann et al (43) and Patt and Maloney (58, 59).

A study of Tables 3 and 4 shows that there is a discrepancy between flow rates from M_1 and M_3 determined by mitotic index or H^3TDR labeling data. This is perhaps not surprising since the computations are already dependent upon the relative abundance of cells in each cytologic phase.* The wide variation in normal bone marrow differential counts is well known. For comparative studies it seems obvious that H^3TDR labeling and mitotic counts must be made on the same individuals and compared within this individual. Since almost all biologic observations have a statistical variation between subjects one probably needs to measure mitotic time, DNA synthesis time, labeling and mitotic indices in the same individual. So far as conscious patients are concerned this becomes impractical.

The presence of a mitosis means that within the mitotic time the cell will have divided - the population is increased by one. However, division may follow DNA labeling by many hours (as long as the sum of maximum DNA synthesis, R_2 and mitosis) thus in a system where proliferation and maturation go in parallel division may occur in the next cytologic phase. In this

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* I_L and I_M performed on different subjects.

respect, the ultimate relative production at M_3 of 322 for mitotic index and 276 for DNA labeling are not so widely divergent as at earlier stages.

Another facet of interest in the marrow flow of cells is the apparent difference between M_3 production of 276 and input into M_4 of 134. This "myelocyte sink" is a key point in the Patt and Maloney model for granulopoiesis (58). Does this "myelocyte sink" exist? Certainly our human data and their canine data show an apparent "sink" when one makes computations with a DNA synthesis time of 5 hours. With our human data the "sink" disappears if the DNA synthesis time is increased to about 10 hours. Certainly DNA synthesis times of this duration exist in some human tissues. However, of even more importance is the question of the reliability of metamyelocyte (M_4) replacement by influx of labeled cells from M_3. Bond et al (7) clearly pointed out that this type of computation assumes that no unlabeled cells are entering during the period that one measures the apparent replacement. It is pertinent in human beings that the smallest class of M_3 has a low labeling index of about 0.20 (19). It is also clear that the sum of R_2 and mitosis is short ($\sim$ 3 hours) hence labeled cells will enter M_4 after this period. They do (19,27,57). The maximum labeling is attained only after 2-3 days. Similarly the labeling in M_3 rises from 0.20 to 0.72 approximately the same as labeling in M_4 after 2-3 days. One has two choices either small myelocytes which are unlabeled after a "flash" label, die at the M_3 level or they mature to metamyelocytes. In the event that the latter occurs in part unlabeled cells are entering with labeled cells and the M_4 replacement is significantly greater and the basis for the "myelocyte sink" evaporates. At the present time no experimental way is seen out of this enigma. The Patt and Maloney model for granulocyte kinetics remains intriguing

(59) as well as their concept of "death controlled" production of granulocytes. It's proof lies in an experimental eradication of the underlying enigmata. The reader is referred to Patt's imaginative and logical discussion of granulocyte kinetics for further details. The remainder of this discussion on granulocytes will focus upon direct observation on granulocyte emergence from the bone marrow and disappearance from the blood.

Emergence from the Marrow:

The variation in emergence time from the marrow is illustrated in Figure 5 from Fliedner et al (29). The range is from 96-144 hours for human beings in "normal steady"equilibrium. It is reduced to 48 hours in acute infections. The slope of increase in index of labeling has been similar in all except two patients. The replacement rate is 0.02 per hour based on influx of labeled cells.

Mauer et al (52) have assumed that cells are lost randomly from the blood with a half time of 6.6 hours. Fliedner et al (30) have demonstrated a random loss into one area, the oral epithelium. Labeled granulocytes appear in the saliva of normal human beings immediately after their appearance in the peripheral blood thus providing direct cytologic and autoradiographic proof of a random loss from the blood irrespective of the age in the blood. One can then accept with some confidence the half time of 6.6 hours. With this half time, the fractional replacement per hour, λ is

$$\lambda = \frac{0.693}{T\frac{1}{2}} = \frac{.693}{6.6} = 0.105$$

Since the "steady state" demands equal influx and efflux there must be four times as many unlabeled as labeled cells entering during this initial period of emergence.

The Loss of Granulocytes from the Blood:

The evidence for a dual loss of granulocytes from the blood has been presented elsewhere (28). It is summarized as follows. Pyknotic granulocytes are observed in small numbers in the blood (1.5 to 8 per 1000 granulocytes). Label appears in the pyknotic cells about 30 hours after it is first observed in mature granulocytes in the blood. Ascending curves of labeling of normal and pyknotic cells thereafter remain parallel hence the random disappearance of granulocytes from the blood is presumably truncated by senescence at about 30 hours after emergence into the blood. The second mechanism of loss was implied to be random from the work of Mauer et al (52) and a definite random loss was observed cytologically by Fliedner et al (28) mentioned above. Thus there are two losses - random and senescent. This can be shown schematically in Figure 6 a, b from reference (28). In this figure

N = number of normal granulocytes in the blood

P = number of pyknotic granulocytes in the blood

λ_1 = fraction of N becoming P in unit time.

λ_2 = fraction of P leaving circulation unit time

λ = fraction of N leaving circulation unit time

$\lambda_1 + \lambda_3$ = fraction of granulocytes being replaced per unit time.

Degradation of N to P is a terminal process not a random process. The equations for determination of the time for disappearance of pyknotic cells are published (28). With a half time of 6.93 hours* in the blood for N granulocytes, the half time in the blood for pyknotic cells is 15.9 minutes.

From the preceding it becomes clear, despite the enigmata stated, that knowledge of the flow pattern and flow rates through the marrow pools, release

---

* Chosen instead of Mauer's 6.6 hours to simplify arithemtic.

from the marrow and disposal from the blood is accumulating rapidly. The gaps in knowledge do not interfere seriously with a comprehensive picture of granulocytopoiesis from a quantitative standpoint. One can expect with confidence that these gaps will be plugged in the near future.

LYMPHOPOIESIS:

Much is known about the behavior of lymphocytes. This very knowledge of the intricate pathways of migration and recycling prevents an analysis today that is comparable to what has been presented for erythropoiesis and granulopoiesis. The complicating features that confounds the observer are the migration pathways:

a. Lymph nodes to blood to lymph nodes (33,70),

b. Thymus - blood - spleen (25),

c. Lymphocytes to marrow and back? (41).

Furthermore, lymphocytes consist of a family - not a single entity - yet they migrate together apparently and live in the same house when at home. The small class is long lived - some perhaps as long as 100 days (49). The nature of loss-senescence or random or both is vague. The larger cells are shorter lived. The small cell is not an end cell. It can, when given the right conditions, transform and divide (50). The small lymphocyte apparently participates in the primary response, becomes "imprinted" with an immunologic destiny, migrates to lymphocytic tissue, establishes clones and undergoes transformation and proliferation (34). Presumably these develop antibody producing nodules. Clearly, the secondary nodules (germinal centers) develop only with the anamnestic response (12).

The preceding facts of a mixed family, diverse life spans and functions and primarily the migration patterns with apparent aimless recycling (nodes-lymph-blood-nodes) that cannot be quantitated precludes a critical analysis of the kinetics today. The same, but nameless cells, traverse the same anatomic observation window and confounds the interpretations of the observer.

New approaches to the study of lymphocytopoiesis are necessary. We have perfected extracorporeal irradiation of the blood (13, 21, 22) to deplete lymphocytic tissue without direct radiation injury to the formative tissue as a means to study the stressed system, function control and rates of proliferation. Depletion has been accomplished (13). Whether this new "dodge" will be ultimately effective in "cracking the tough nut" of lymphopoiesis remains to be seen.

Description of Cellular Flow Patterns and Rates:

Progress is being made at an accelerated rate. Useable and hence satisfactory numbers for the time parameters of the cell cycle in hemopoietic tissues, flow rates through catenated cytologic compartments, steady state and maximum production rates and peripheral loss from the blood either exist or will be available in sufficiently refined form in the foreseeable future except for lymphocytopoiesis. The rapid recent advances were made possible primarily by the development of a specific DNA label (37) that produces high resolution autoradiographs so that single cell populations could be observed directly.

Speculations on Regulatory Mechanisms-Possible Future Directions of Research:

A useful starting point for general discussion revolves around the random loss of granulocytes from the peripheral onto oral epithelium and presumably

other epitholia. Could this random loss result from chemotactic attraction by the contaminating bacteria? If so granulocytes will not be found upon the epitholia of germ free animals. In this case, disappearance from the blood may be purely the result of senescence. Those who have germ free animals can test this hypothesis easily by simple direct experiments.

Peripheral loss is followed promptly by replacement from marrow reserves. Truly huge amounts can be replaced rapidly to wit Craddock's (15) and others (3, 38) remarkable experiments on leukocytophoresis. Insight into the mechanism of release is being accumulated systematically by Gordon and associates (31, 32). Increased blood flow through the marrow and decreased granulocyte content of affluent blood "dumps" reserve granulocytes of the marrow into the periphery. Since there appears to be a unidirectional flow of granulocytes from marrow parenchyma into the closed sinusoidal system that is part of the blood stream, one can imagine that a "communication" exists between intra and extra- sinusoidal granulocytes that detects differences in concentration. As the difference in concentration increases more marrow granulocytes are diverted for action and the flux across the sinusoidal membrane increases. If blood flow through the marrow simultaneously increases the total granulocyte input into the blood will be further enhanced. The preceding line of reasoning is compatible with observations on leukopheresis (3,15,38) and leukocytosis producing agents (31,32).

As the reserve of granulocytes in the marrow acceptable to the blood is depleted, input to the non-proliferating compartment increases. Whether this is due to turning off the alleged "death" at the myelocyte "sink" and/or to actual increase in intrinsic production rate at a more immature level remains vague.

Certainly Patt's concept of "death controlled" production of granulocytes is deserving of serious thought and direct experiment to prove unequivocally the existence of the "sink" and its elimination by experimentally increased peripheral consumption of granulocytes.

The failure to isolate the counterpart of erythropoietin to date is not reason to deny its existence nor to believe that "death control" is the sole regulatory mechanism. They are not mutually exclusive. If there is a specific stem cell for granulocytopoiesis an intramedullary "death" function may decelerate production. Elimination of "death" by increased peripheral use may be assumed to remove a governor which lets the system go "flat out" to a maximum possible production rate. Decreased peripheral use would then bring the system back to "death" control. I like the idea of the death control but it is still possible to explain away the myelocyte "sink". This latter demands unequivocal, rigorous experimental proof.

In respect to granulocytopoiesis other areas deserving of research ~~is~~ are the nature of the chemical changes within the maturing granulocyte precursors that:

1. Turn off further cell cycling at the myelocyte level.
2. Permit granulocytes to enter the blood stream or be called into the stream.
3. Lead to peripheral senescence and loss without functional use.
4. Draw cells out of the circulation or permit them to be drawn out.

The stem cell pool, its assumed "size conscious" sensing system, nature of communication between stem cells and identity of the stem cell(s) are problems deserving of further discussion. However, direct evidence to support

ideas is sorely lacking. Stem cell(s) do migrate through the blood (8,30) and the only reliable source for all stem cell(s) is the marrow (4). Evidence accumulates that the small marrow lymphocyte is the probable stem cell (23,26). If the small marrow lymphocytes proliferate within the marrow one should see "flash labeling" and mitotic figures in marrow lymphocytes. This is a great rarity. However, labeling increases with time hence there is migration to the marrow (41, 70). Do these immigrants become stem cells? First all cells are presumed to have the total genetic code locked within their DNA. Differentiated cells have all but the necessary bits suppressed. Since the stem cell(s) are only found in the marrow and do not seem to proliferate in the marrow one is inclined to imbue the marrow with the capability of imprinting the lymphocyte immigrants with stem cell properties. This would be consistent with Yoffey's ideas (70). Perhaps chance residence near an island of hemopoiesis unlocks the capability of proceeding down the specific pathway of differentiation. This line of reasoning is unfounded except by vague embryologic analogy to progressive differentiation from conception onwards. For the rest of the argument it makes little difference whether the imprinted stem cell capability is specific or common. The migration in the blood through the kidney may have specific importance and certainly would improve the probability of erythropoietin penetrating into the activated stem cell. It may be more than fickleness of evolution that in earlier vertebrates hemopoiesis is closely related to renal tissue. Thus one conceives of three possible steps in the mammal for commencement of erythropoiesis in the normal steady state.

a. Lymphocyte immigration to the marrow.

b. Imprinting of erythropoietic stem cell capabilities.

c. Uptake of erythropoietin at the kidney and return to the marrow.

In the case of severe hypoxia, the kidney (and perhaps other areas) dumps erythropoietin into the circulation and the probability of penetration into a stem cell wherever it may be increases. The intravenous injection of exogenous erythropoietin reduces the assumed need for migration through the kidney. The suppression of erythropoiesis by hypertransfusion is very intriguing and may act through increased oxygen transport suppressing erythropoietin production so that the proliferating erythropoietic precursors disappear by maturation leaving imprinted stem cells awaiting erythropoietin or, increased oxygen transport to the marrow suppresses cell cycling. In this respect it is of interest that 100% oxygen in vitro suppresses DNA synthesis in erythropoietic precursors (Rubini and Cronkite unpublished).

An analogy between Hoffman's ideas presented at this Symposium on hormone action and erythropoietin may be made. Presumably RNAase is incomplete. A polypeptide penetrates into and completes the enzyme and the rate of RNAase activity becomes maximal. An extension of this concept to erythropoietin follows.

The initial step in erythropoietic differentiation will be considered marrow imprinting of a "lymphocyte immigrant" which sets the stage by unlocking the potential flow of information within the genome for hemoglobin synthesis. Then synthesis of messenger RNA and transfer of the code to the ribosome becomes possible. Erythropoietin penetrates into the cell and completes the synthetic complex at the ribosome level thus either starting or accelerating the production of hemoglobin to a maximum rate if all substrates are available. The actual sequence of assumed events is not clear. Finally, RNAC (or some process proceeding in parallel) begins to repress the

hemoglobin synthesis, turns off cell cycling and kicks out the shrunken normoblastic nucleus. This concept of erythropoietin action would place it in the category of a classical hormone - a determinant of rate of specific synthetic processes.

This highly speculative three step model for erythropoiesis appears to be consistent with the experimental observations and ideas of Stohlman and Lajtha. The attractiveness of this working comprehensive model for hematopoiesis lies within the fact that it attempts to combine observed facts, current ideas on protein synthesis, hormone action, and chemical communication systems within cells. It's final charm to me is the fact that experiments become obvious by which one can test the validity of some of the steps. For example, Quastler's hypothesis (62) can be tested in principle: "ther some inducer should be found attached to ribosomes in the process of induction. This possibility has not been explored. The number of inducer molecules so bound should be quite small - still, a negative result under sufficiently sensitive conditions would disprove the hypothesis." It appears worthy for someone to attempt to suppress erythropoiesis by hypertransfusion, administer large amounts of erythropoietin and see if it can be recovered from the ribosomes of a stem cell population using Cudkowicz's method of isolating stem cells in the hypertransfused rodent (23).

Both Stohlman and Lajtha relate hemoglobin synthesis to ultimate cessation of mitosis and extrusion of the late normoblastic nucleus. Lajtha remains ambiguous as to whether hemoglobin content (MCH), hemoglobin concentration (MCHC) or decreasing RNA content is the primary repressant for mitosis. Stohlman clearly implicates MCHC and is able to predict macrocytosis, microcytosis

and explains his earlier ideas on skipped divisions. In reality, the influence of cell differentiation upon cell proliferation goes back to at least 1905 (see Hughes, 36), Weiss (69) explicitly states "Cellular differentiation and cellular multiplication are two processes which, if not mutually exclusive, are nevertheless markedly antagonistic in their tendencies". Hemoblobin is an obvious sign of differentiation. Its concentration may well be related to initiation of a series of chemical communication events within a normoblast that suppress synthesis via the ribosomes and feeds back information to repress nuclear (genic) control of hemoglobin and other parallel synthetic processes via the messenger RNA ribosome pathway. See Jacob and Monod (39) and Quastler (62) for a theoretical discussion on communication systems applied to nuclear control of synthetic processes.

The idea that erythropoietin enters stem cells and differentiated red cell precursors and reacts with an unidentified component to produce (induce) more enzymes capable of hemoglobin synthesis is most attractive. Thus hemoglobin synthesis rate and differentiation rates are a function of available erythropoietin, thus shortening the time for emergence of red cells, an observed fact.

Lajtha's and Stohlman's observations are doubly important because they point up the possibility of finding chemical entities that participate in the regulation of cytoplasmic nuclear interactions as a means of blocking or unblocking the natural function of a cell to divide. Thus one is encouraged to look more carefully for chemical changes in the cytoplasm of maturing cells in other cell renewal systems as a suppressant of cell cycling which may be a major factor in maintaining orderly "steady state" growth.

A disconcerting observation is the apparent absence of erythropoietin

secretion in the llama which normally resides at altitudes that produce polycythemia in other mammals (personal communication from C. Reynafarjes). If verified this exception to a generality may be of great evolutionary importance. It may imply that the erythropoietin mechanism develops only in those mammals that need it or that it has been lost or supplanted by other mechanisms in those animals that live normally under low oxygen tension. A study of erythropoietic mechanisms in these animals at sea level and high altitude may be most rewarding.

In concluding, scientific cytology commenced in reality with the brilliant observations by and through crude microscopes by Van Leeuwenhoeck about 260 years ago. We are fortunate today to be witnessing the accomplishments of a vigorous and publicly supported scientific community apply modern tools and amass a vast store of factual information on chemical and physical processes in and between living cells. In parallel this information is being correlated and blended into comprehensive plausible pictures of living processes. One can have easily the illusion that all of life's secrets are springing from Pandora's box (the blessings not the ills). It is truly exciting to observe the productive wedding of so many scientific disciplines with the resultant highly imaginative and ingenious generalities on behavior of the cell. However, in all humility, one must grant that the end of research on cells is not near and that the future of cytology will be longer and more brilliant than the past.

It has been most enjoyable to be permitted these correlations of facts and flights of fancy. May future experiment confirm or destroy these thoughts. I can think of no better concluding statement for those who speculate than a quotation from Charles Darwin. "False facts are highly injurious to the

progress of science for they often endure long; but false views, if supported by some evidence, do little harm for everyone takes a salutory pleasure in proving their falseness."

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TABLE 1

Relative Compartment Size of the Nucleated Red Cell Precursors, Mitotic Fraction, Computation of Duration Spent in Certain Cytologic Phases, with Rate within Mitotic Compartments and Flow Between Compartments

	$E_1 + E_2$	E_3	E_4	E_5
Relative Compartment Size	100	210	423	611
Fraction in Mitosis	0.025	0.049	0.056	0
Minimum CTT* hrs.	-	9.5	6.2	
CTT	23	-	-	
Maximum CTT "	-	48.5	19.2	
Generation time hrs.	-	-	9.0	
Birth Rate (relative)**	4.3	17.9	41.2	
Influx	***	4.3	22.2	30.5****
Efflux	4.3	22.2	63.4	

* Mitotic time of 0.58 hrs. from Odartchenko et. al (37)

** Birth rate = $\frac{\text{number in mitosis}}{\text{mitotic time}} = \frac{\text{fraction in mitosis x relative number}}{0.58}$

*** Assume no stem cell input

**** Estimated from 5.3% per hour replacement from Bond et al (3)

TABLE 2

H^3TDR Labeling of Erythrocytic Precursors, Fraction Labeled, Time for R_1, DNA Synthesis, R_2, Mitosis and Proliferation Rates. Observations on Human Beings Except where Indicated.

	$E_1 + E_2$	E_3	E_4	E_5
Relative number	100	210	423	611
Fraction labeled (I_L)	~1.0	0.78	0.33	0
Time for R_1 hrs.	-	-	1-3	
Time for S	-	-	7	
Time for R_2	-	-	1-2.5	
Time for Mitosis	-	-	0.58*	
Time for Extrusion of E_5 Nucleus	-	-	-	8.5-12*
Generation Time	24	?	9-10*	
Halving time for grain Count	24	~ 24	~ 24	
Birth Rate	14.3	23.2	20.0	
Influx	**	14.3	37.5	35.5
Efflux	14.3	37.5	57.5	

* Observations or computations in the dog.

** Assume no stem or minimal cell influx.

TABLE 3

Relative compartment size of the granulopoietic precursors, mitotic fraction, computation of duration spent in certain cytologic phases, birth rate within cytologic compartments and flow between compartments. Mitotic time (t_M) 0.58 hrs. ( )

	M_1	M_2	M_3
Relative compartment size*	100	337	1630
Fraction in mitosis	0.025	0.015	0.011
Minimum CTT (hrs.)		26	37
Ctt	23.3		
Maximum CTT "		78	126
Generation time	?	?	?
Birth rate **	4.3	8.8	309
Influx **	?** *	4.3	13.1
Efflux **	4.3	13.1	322.1

* Data from reference ( ).

* Method of computation shown in Table 1.

** Stem cell influx considered negligible .

TABLE 4

H^3TDR Labeling in Granulocytic Precursors, Labeled Fraction, Duration of Various Phases of Cell Cycle and Transit in a Single Patient.

	M_1	M_2	M_3	M_4	M_5	M_6
Relative compartment* Size (N)	100	286	4830	4460	7850	8075
Fraction labeled (I_L)	.85	.65	.23	0	0	0
Time for R_1 hrs.				-	-	-
Time for S " **	?	?	?	_	-	-
Time for R_2	?	?	~ 2.5	-	-	-
Time for mitosis hrs.	~ 0.58	0.58	0.58	0.58	-	-
Generation time (t_G)	?	?	?	-	-	-
Halving time for grain count	~ 24	~ 60	~ 54	-	-	-
Birth rate ***	17	37	222	-	-	-
Influx ***	?	17	54	134***	134	134
Efflux ***	17	54	276	134***	134	134

* From marrow differential in whom labeling studies were performed ( ).

** Time for synthesis assumed to be 5 hours as determined Patt ( ) in dog.

*** Based on metamyelocyte replacement of 3% per hour.

**** Method of computation same as shown in Table 1 for mitotic index.

Figure 1. Schematic presentation of proliferation, maturation, function and death in serially connected cytologic compartments.

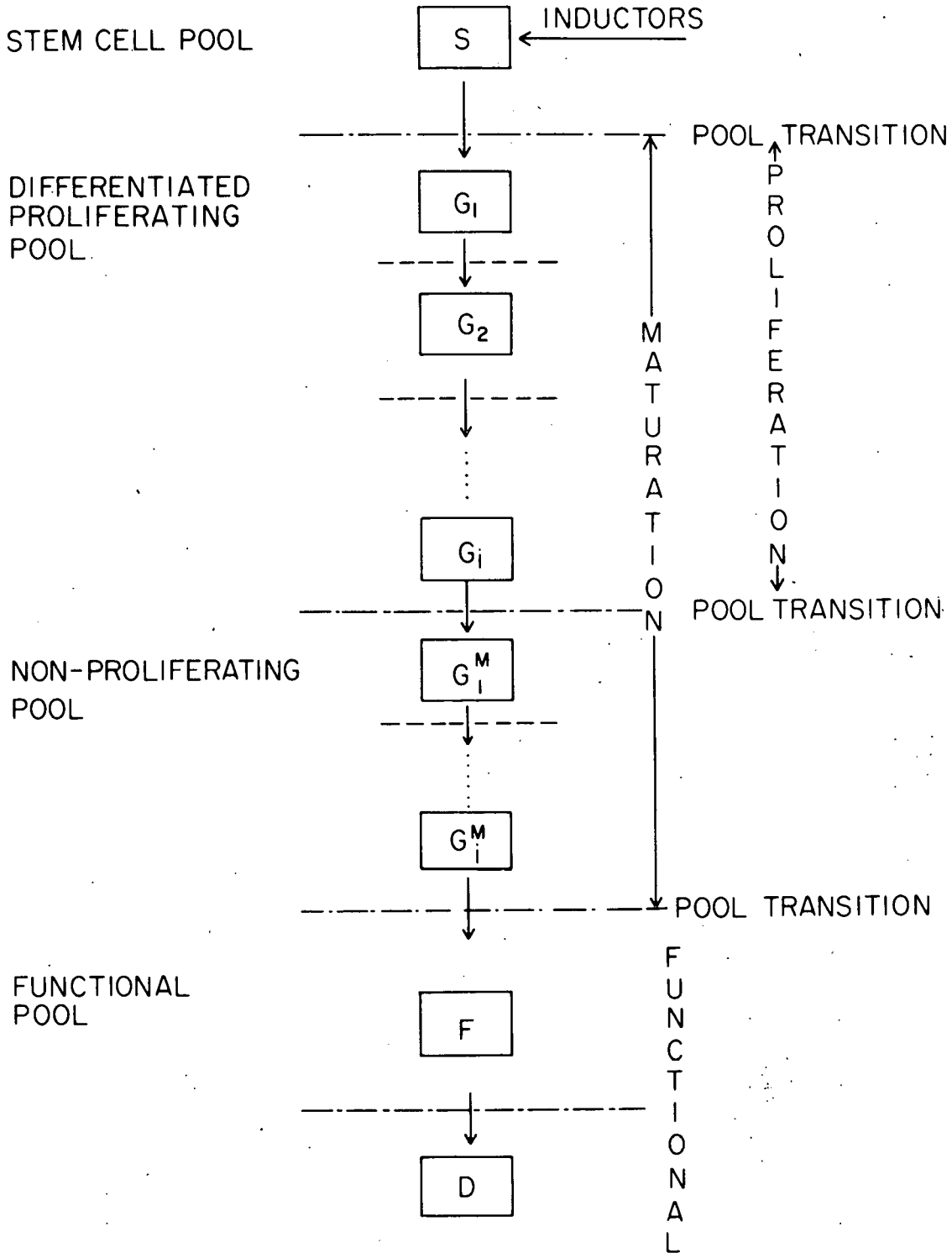
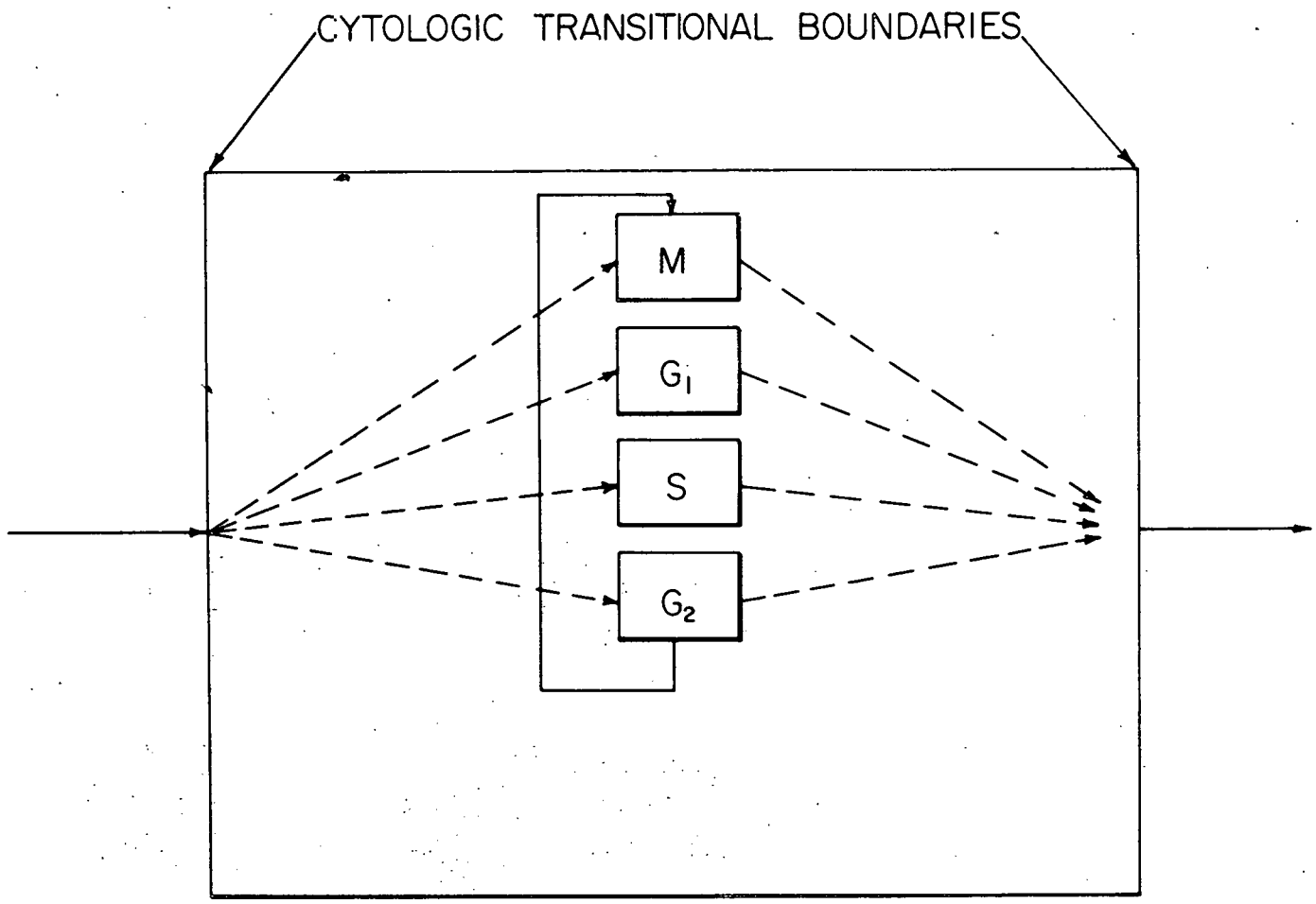

STEM CELL POOL
S
INDUCTORS
POOL TRANSITION
DIFFERENTIATED PROLIFERATING POOL
G1
G2
Gi
MATURATION
PROLIFERATION
POOL TRANSITION
NON-PROLIFERATING POOL
G1M
GiM
POOL TRANSITION
FUNCTIONAL POOL
F
D
FUNCTIONAL

FIGURE 1

Figure 2. Schematic presentation of a proliferating cytologic compartment within the differentiated proliferating pool. There is no capacity for self- perpetuation- only multiplication. Entering cells may cycle one or more times or proceed through only a part of the cell cycle depending upon the rate of maturation or time before they resemble the next cytologic class. Diagram from H. Quastler (63).



FINE STRUCTURE OF A PROLIFERATING COMPARTMENT

FIGURE 2

Figure 3. Schematic presentation of flow of erythropoiesis from the stem cell, through the proliferating pool, non-proliferating pool, and blood to ultimate death by senescence. Alleged points of action of erythropoietin are indicated.

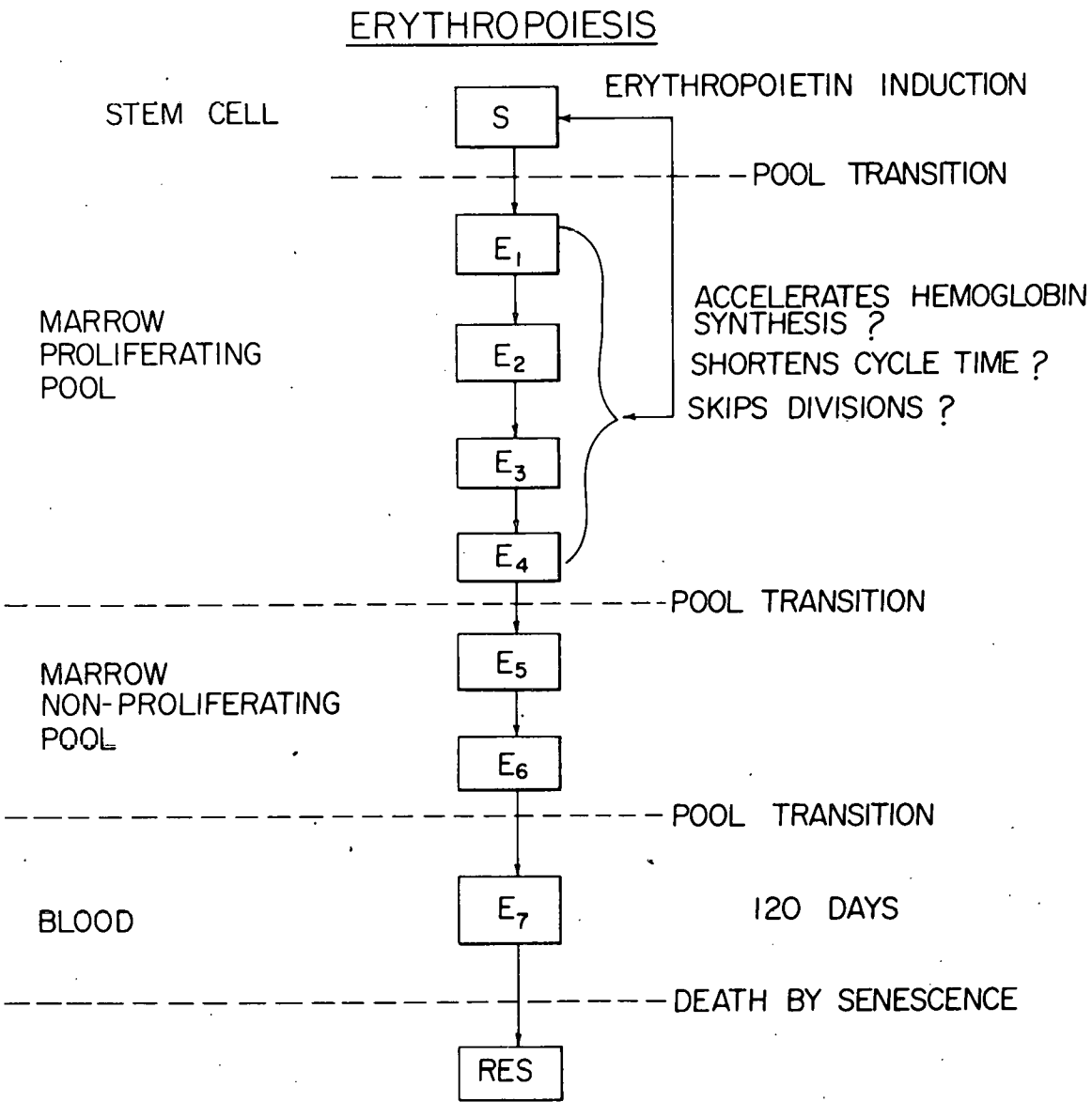

ERYTHROPOIESIS
STEM CELL
S
ERYTHROPOIETIN INDUCTION
POOL TRANSITION
E1
E2
E3
E4
MARROW
PROLIFERATING
POOL
ACCELERATES HEMOGLOBIN
SYNTHESIS ?
SHORTENS CYCLE TIME ?
SKIPS DIVISIONS ?
POOL TRANSITION
E5
E6
MARROW
NON-PROLIFERATING
POOL
POOL TRANSITION
E7
BLOOD
120 DAYS
DEATH BY SENESCENCE
RES

FIGURE 3

Figure 4. Schematic presentation of the total life cycle of the granulocyte. For definition of λ's see text.

SCHEMATIC PRESENTATION OF THE LIFE CYCLE OF THE GRANULOCYTE

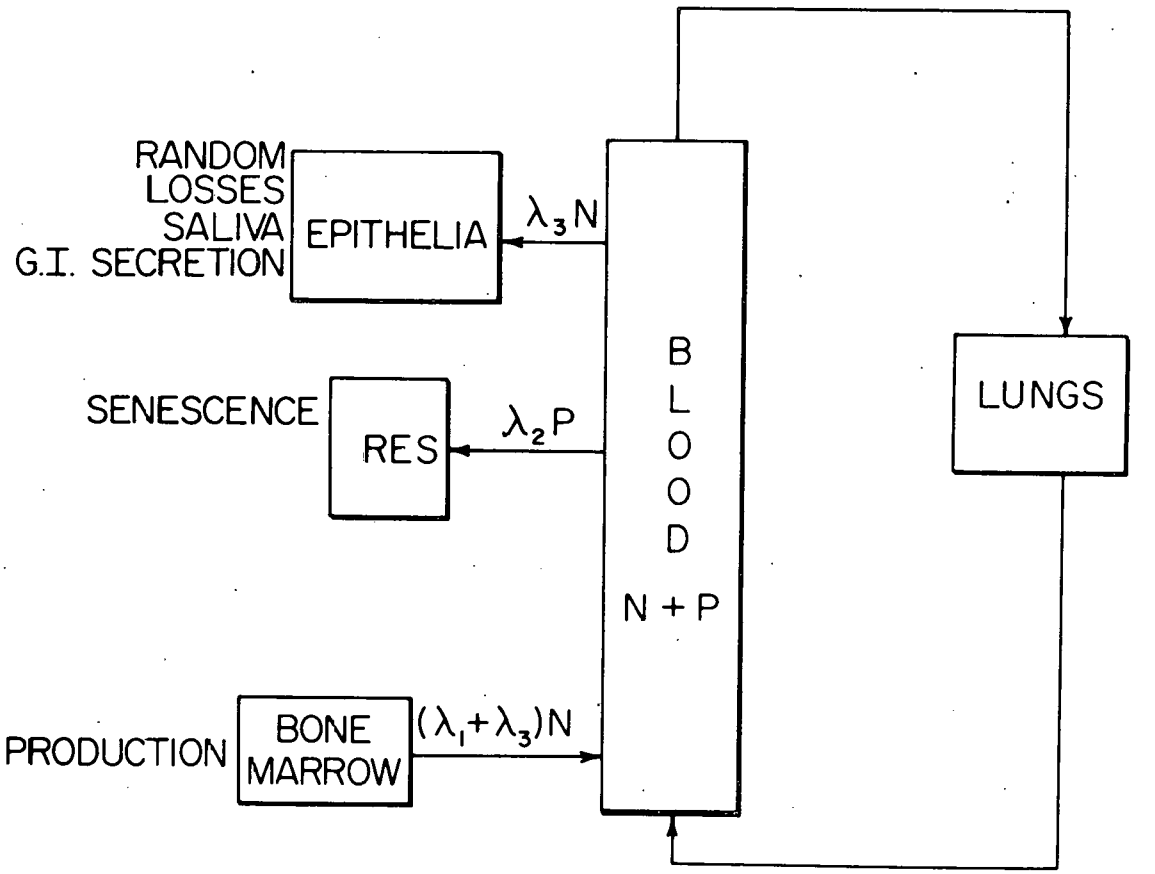


FIGURE 4

Figure 5. The emergence time of blood granulocytes from the marrow (from Fliedner et al, 29).

11-1-99-62

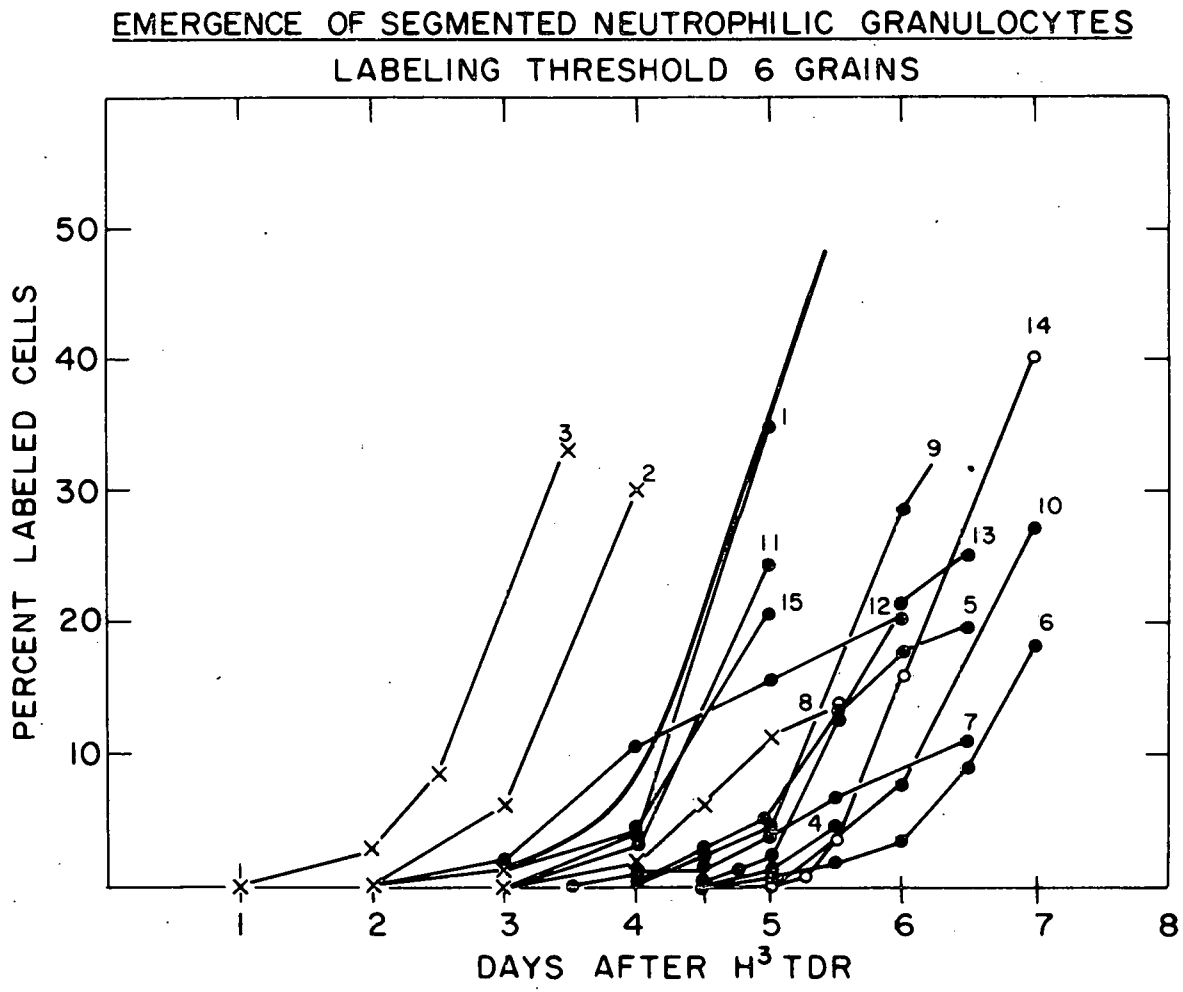
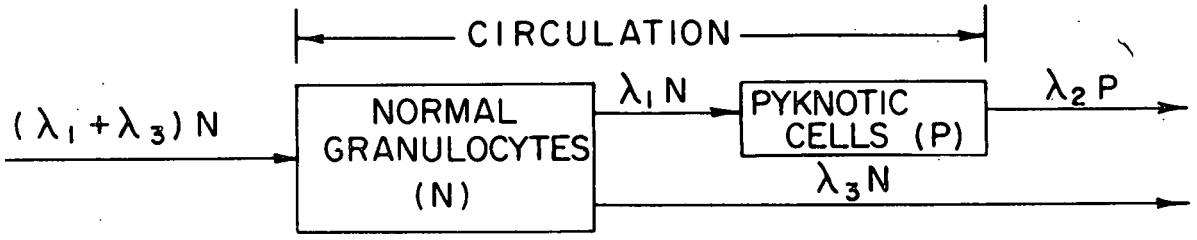

EMERGENCE OF SEGMENTED NEUTROPHILIC GRANULOCYTES
LABELING THRESHOLD 6 GRAINS
PERCENT LABELED CELLS
50
40
30
20
10
1
2
3
4
5
6
7
8
DAYS AFTER H³ TDR
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

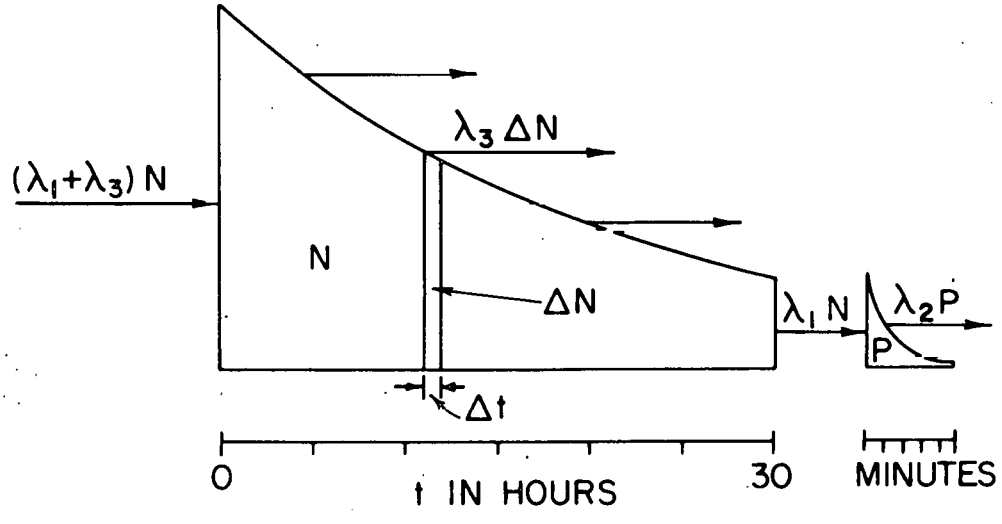
FIGURE 5

Figure 6. Schematic presentation of loss of granulocytes from the blood. 6a shows the compartmentalization and 6b a graphic representation of exponential disappearance (age distribution in blood) truncated by senescence at 30 hours (from Fliedner et al, 26).

1-183-6a



6a



6b

FIGURE 6

52

53