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An Analysis of Leukocyte Radioactivity Curves Obtained with Radioactive Diisopropylfluorophosphate (DFP^{32})

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THE REACTION of diisopropylfluorophosphate (DFP) with the amino acid group that forms the reactive center for several enzymes is shown in figure 1. The DFP molecule forms an irreversible bond with these enzymes, releasing HF and yielding a diisopropylphosphate-enzyme molecule. When the enzyme is broken down, diisopropylphosphate (DIP) is released. DIP is nonreactive and is promptly excreted.

The irreversibility of the bond and the lack of reutilization of the breakdown product suggested the use of this molecule, when tagged with P^{32}, as a cell label.[2] We have been concerned with the use of DFP^{32} as a granulocyte label. This can be done if certain criteria are met. First, since the enzymes that are bound by DFP are present in red cells, plasma, and platelets, isolation of the leukocytes must be complete. Second, of the leukocytes in the blood, only granulocytes must label to a significant degree. Finally, there should be no measurable effect of the DFP on granulocyte function in the concentration used to label the cells. The evidence that these criteria have been fulfilled has been presented.[1]

Granulocytes have been labeled both in vivo and in vitro by DFP^{32}. In vivo labeling has been done by the intravenous injection of DFP^{32}. Granulocytes are labeled in vitro by drawing whole blood into plastic bags with ACD solution as an anticoagulant and incubating the blood for one hour with DFP^{32}. The labeled blood is then returned to the subject. The methods for isolation of the leukocytes and determination of their radioactivity have been described.[1]

A representative leukocyte radioactivity curve obtained after the intravenous injection of DFP^{32} is shown in figure 2. The lower curve represents the radioactivity as CPM per mg. of leukocyte nitrogen. The upper curve is drawn through the logarithms of the leukocyte radioactivities.

During the first two days after the injection of the DFP^{32}, there is a rapid decrease in leukocyte radioactivity (phase I). The leukocyte radioactivity then remains fairly constant until the 11th day (phase II). A final decrease in radioactivity (phase III) follows. By about the 24th day, no radioactivity can be measured in the leukocyte samples.

The results obtained in two subjects after in vitro labeling of their granulocytes are shown in the lower portion of figure 3. The leukocyte radioactivities are expressed as the logarithms of the CPM per mg. of nitrogen. For comparison, in the upper portion of this figure the logarithms of the leukocyte radioactivities obtained during phase I, after in vivo labeling of leukocytes in two subjects, are

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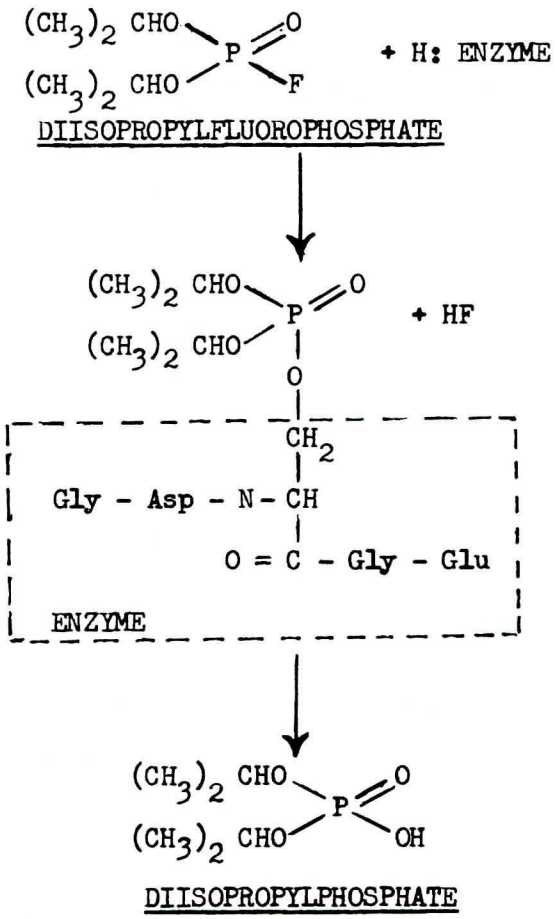

FIG. 1.—Model of the reaction of DFP with the active serine group of an enzyme. Gly, Asp, and Glu refer to the amino acids glycine, aspartic acid, and glutamic acid (from Athens et al.: Blood *14:* 303, 1959).

shown. The open circles are the logarithms of the values obtained by subtracting the mean phase II values from phase I.

If the curve obtained after in vitro labeling is compared with the curve obtained after in vivo labeling, a clear difference can be seen. Analysis of the curves makes it appear likely that granulocytes in sites other than the blood are being labeled by the in vivo method.

To assist in the analysis of the curves, kinetic models were set up on an analog computor and tested for feasibility. The first step was to define the possible types of cell compartments that might be present in any granulocyte kinetic model. These types are represented in figure 4. In this figure, the cells entering the compartments are considered to be unlabeled, whereas those in the cell compartments are considered to be labeled maximally at zero time.

A type I compartment is a simple, miscible pool of cells that leave in a random fashion. The radioactivity expected in the cells coming from this pool after time t_0 would reflect the average radioactivity of the pool itself and would decrease

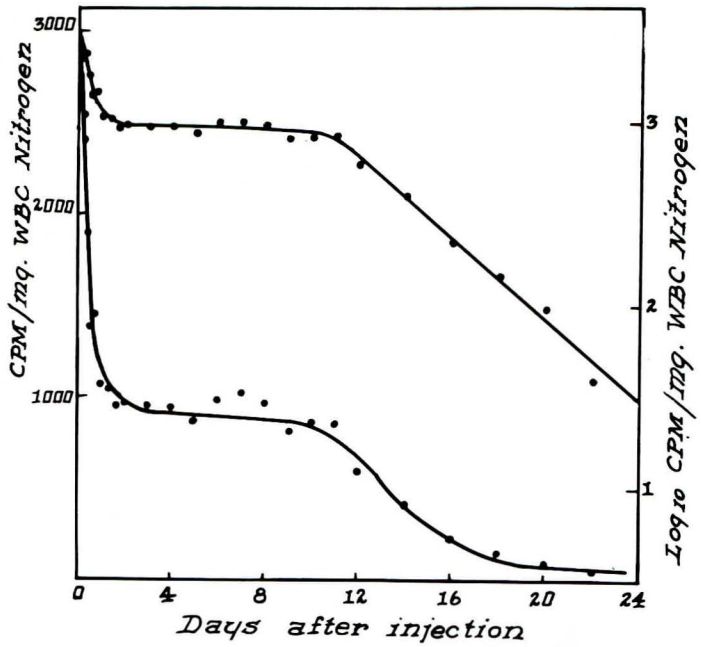

FIG. 2.—Arithmetic and semilogarithmic curves of the leukocyte radioactivities obtained after the intravenous injection of DFP^{32}. Each curve represents the mean of seven individual curves (From Athens et al., Ann. N. Y. Acad. Sc., in press).

TABLE 1.—*Comparison of the In Vivo Phase I with the Disappearance of In Vitro Labeled Cells*

Groups	No. Subjects	Mean T½ ± S.E. (Hours)	P
In Vivo	30	6.4 ± 0.37	>0.05
In Vitro	20	7.4 ± 0.33	

in an exponential manner. The mathematical expression of this decrease is given below the model.

A type II compartment would be expected if cells in a particular phase must spend a definite time in the phase before release. Thus, the cells would behave as if they were coming down a tube, aging as they proceed, pushing before them older cells and being replaced by younger cells. Once the cells in the tube had been labeled, there would be no change in the radioactivity of cells leaving the tube, until the youngest cells at the time of labeling had left and were replaced by cells that were not in the tube at the time of labeling. At this time, the radioactivity of the cells leaving the tube would suddenly fall to zero. The mathematical expression of the radioactivity of the cells leaving the tube is given below this model. Time t_1 is the time when the last labeled cells leave the tube. From t_0 to t_1 is the transit time through the tube. A sample of blood drawn for analysis at any time would reflect the average concentration of label in the pool and would decrease linearly with time.

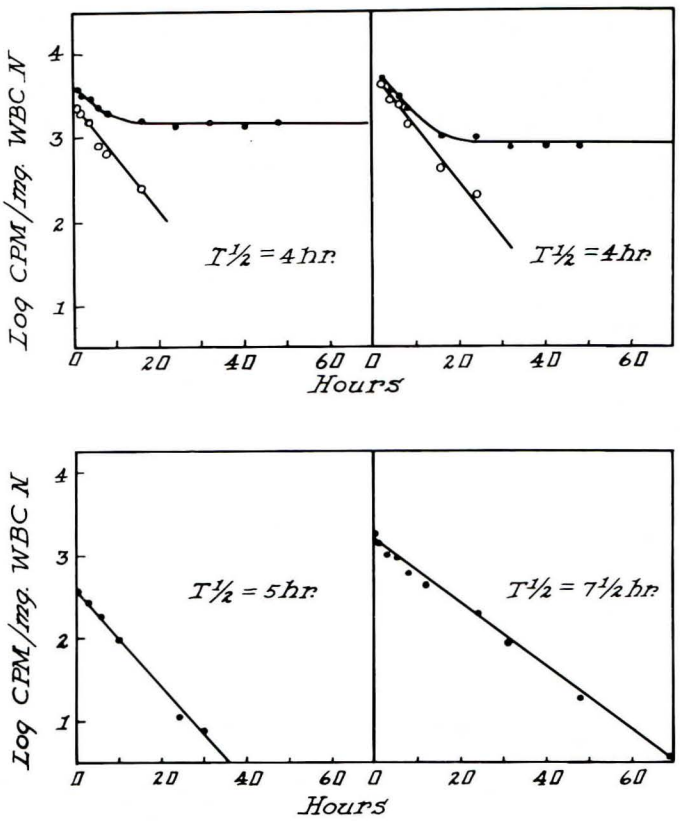

FIG. 3.—Comparison of the in vivo phase I curves (*top*) with the disappearance of in vitor labeled granulocytes.

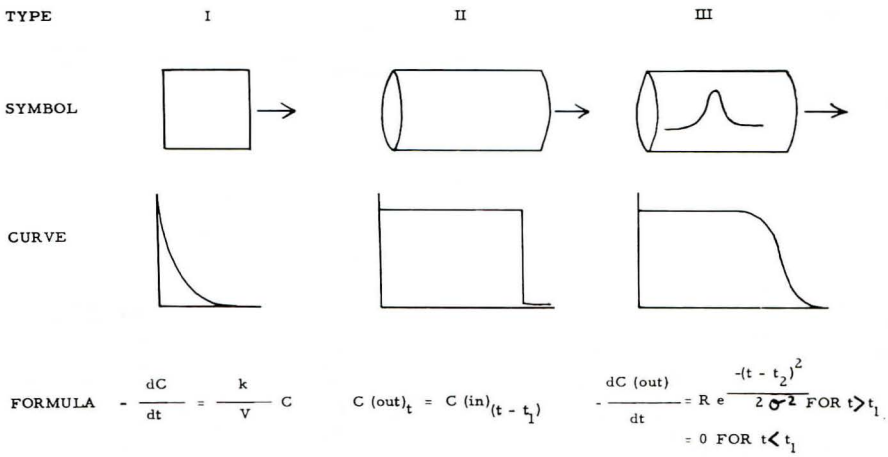

FIG. 4.—Types of cell compartments that may be present in a granulocyte kinetic model

The type III compartment is similar to the type II compartment, except that there is some degree of random distribution of the transit time through the tube. Thus, after the cells in the tube had been labeled, no change in the radioactivity of the cells leaving the tube would be expected until time t_1, the minimum transit

time. The rate of decrease would become maximal at time t_2, when most of the labeled cells were being replaced by unlabeled cells. Some radioactivity would remain in the cells coming out after this time because of mixing of lagging labeled cells with the unlabeled cells. The mathematical expression of the radioactivity of cells coming from this pool is given below the model. R is the maximal rate of change of radioactivity in the emerging cells and occurs at time t_2.

From the radioactivity curves obtained after labeling granulocytes with DFP^{32} in vitro, certain deductions concerning the removal of granulocytes from blood can be made. If granulocytes leave the blood in a random fashion, the blood pool of granulocytes would represent a type I compartment. The fact that the time course of in vitro labeled cells falls exponentially argues for the validity of this concept (fig. 3). If, on the other hand, granulocytes leave the blood compartment in a sequence determined by their time of entry, then the blood compartment would be type II. Since this model would predict a linear decrease in labeled cells, it can be discarded as not compatible with our data. However, the possibility must also be considered that granulocytes are removed from the blood by a combination of these two methods of removal. To investigate this possibility, three such models have been analyzed with the analog computor, but will not be described here. Observations made from these models suggest to us that granulocytes are removed from the blood in a random fashion, entirely or predominantly, independently of the length of time that the cells have circulated in the blood.

From the curves obtained after the in vitro labeling of granulocytes, information concerning the return of granulocytes from the tissues to the blood can be obtained. The model that is shown in figure 5 was subjected to computor analysis. At a rate of return equal to one-half the rate of removal from the blood ($k_2 = \frac{1}{2} k_1$), the lower limits of the size of the tissue pool could be defined. Fair approximation of the data and computor curves were obtained when V_T was allowed to be *greater than* 2.6 times the size of the blood pool (V_B). Upper limits for the return

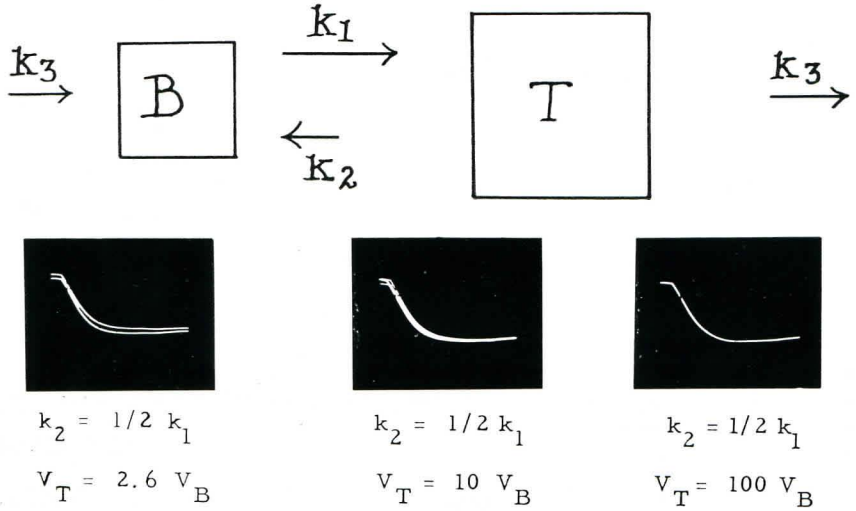

FIG. 5.—Model for possible return of granulocytes from tissues. The method by which the limits can be set for the size of V_T for any k_2 is shown below.

of cells from tissues can be defined by analysis of the in vivo curves, as will be described.

The curves obtained after the in vitro labeling of granulocytes are also of assistance in the analysis and interpretation of the more complex curves obtained following the in vivo labeling of granulocytes. As shown in figure 3 and table 1, the mean half-life of the phase I portion of the curves obtained after in vivo labeling and the half-time of disappearance of in vitro labeled cells are similar. This suggests that phase I of the in vivo curve is due to the removal of granulocytes from the blood. This explanation could be valid if it were assumed that the cells in the blood at the time of the intravenous injection of DFP^{32} are labeled to a greater degree than the cells in the marrow and tissues. This assumption would seem to be resonable, since it would be expected that the circulating cells would be exposed to a higher initial concentration of DFP^{32}. Therefore, in the analysis of the in vivo curves in the analog computor we have assumed that phase I is explained by the removal of highly labeled cells from the blood compartment. On this assumption the initial relative radioactivity of blood granulocytes has been given a value of 100. The radioactivity of the cells in all other compartments has been set at 30. This value was selected because the granulocyte radioactivity levels during phase II are about 30 per cent of the initial value of phase I. Models composed of several postulated anatomic and kinetic granulocyte compartments were then constructed and submitted to analysis in the analog computor. A composite model is shown in figure 6.

M_1 is the pool of mitotically active cells in the marrow. The assumption has been made that the label in this pool decreases in an exponential manner because of cell division and that the level of radioactivity in the cells leaving this pool reflects the radioactivity of the pool. Therefore, this is considered a type I compartment. Cells leaving the mitotic pool (M_1) enter a maturation phase (M_2), from which they normally would not be released until maturation had been completed. M_2 is thus a type II compartment. M_3 represents a storage pool of mature granulocytes in the bone marrow which is ready for release into the blood. The cells contained in this pool, all being mature, are thought to be released in a random fashion, representing a type I compartment. B is the granulocyte pool in the blood. The small "X" compartment in equilibrium with the blood acknowledges the nonhomogeneous nature of the blood pool. If equilibra-

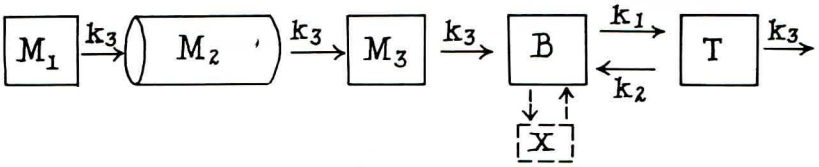

FIG. 6.—A composite granulocyte kinetic model.

1. V_{M_1} is equivalent to V_{M_3} in its effect on the shape of the curve.
2. With M_1 and M_3 present, only the sum of V_{M_1} and V_{M_3} influence the curve.
3. The upper limit of V_{M_1} and V_{M_3} is 10 V_B if $k_2 = 0$.
4. The in vitro curve and phase I of the in vivo curve define the lower limit of V_T for a given k_2.
5. The upper limit of V_T is defined by phase III of the in vivo curve for a given k_2.
6. As V_T increases, V_{M_1} and V_{M_3} must decrease in size.

time. The rate of decrease would become maximal at time t_2, when most of the labeled cells were being replaced by unlabeled cells. Some radioactivity would remain in the cells coming out after this time because of mixing of lagging labeled cells with the unlabeled cells. The mathematical expression of the radioactivity of cells coming from this pool is given below the model. R is the maximal rate of change of radioactivity in the emerging cells and occurs at time t_2.

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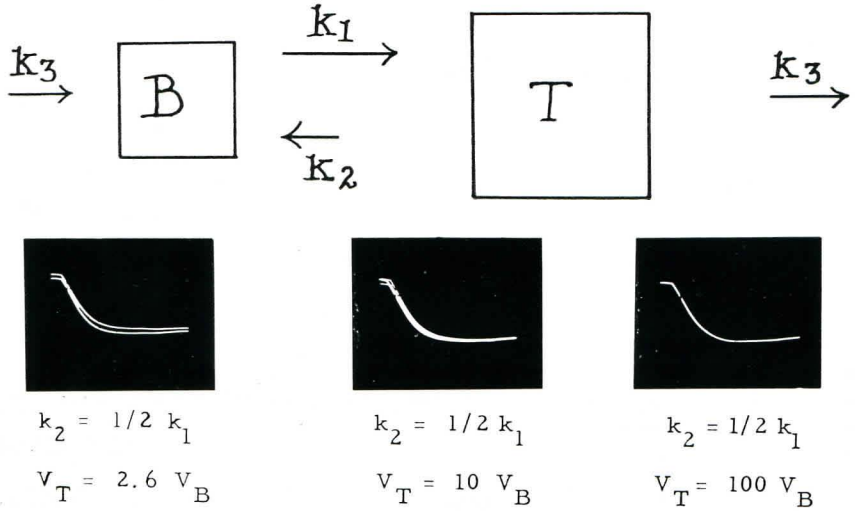

FIG. 5.—Model for possible return of granulocytes from tissues. The method by which the limits can be set for the size of V_T for any k_2 is shown below.

tion were rapid between cells in the circulating blood and the marginal population of granulocytes described by Vejlens,[6] as well as with other possible intravascular pools in the spleen, lungs, etc., the main effect of this "X" pool would be to lead to underestimation of the size of the population of blood granulocytes, since only blood volume and granulocyte concentration are measured. Finally, T is the possible tissue pool of granulocytes that may be in equilibrium with blood granulocytes.

By setting up variations of this basic model on the analog computor and comparing the results with the in vivo data curve, certain generalizations have been made concerning the nature of this granulocyte kinetic model. These generalizations are given in figure 6. Needless to say, the validity of the model depends upon the validity of the basic assumptions already stated.

One of the most interesting observations drawn from this analysis is the limit that can be set on the amount of granulocyte return from tissues to blood. Using analysis of the in vitro curve and the in vivo curve to set limits for the size of the tissue pool and the rate of return, as expressed in generalizations 4 and 5 of figure 6, a maximum return rate amounting to 0.3 of the removal rate is found to be compatible with the data curves.

If a type III compartment replaces the type II compartment that was used to represent the marrow maturation phase in the previous model, a fit can be obtained with the data curve, even if no labeling of the mitotic pool is allowed and the marrow storage pool is disregarded. The standard deviation of random distribution about the mean transit time through the compartment had to be two days. This model is shown in figure 7. The bottom curve represents the random distribution of transit time through the compartment. The middle curve is obtained from the computor model, and upper curve is the data curve.

So far it has not been possible to construct a more exact granulocyte kinetic model from the DFP^{32} data than that presented in figure 6, because of lack of information concerning the degree and extent of marrow labeling. In the near future, it is planned to use tritium-labeled DFP. Demonstrations of the exact distribution of DFP within the granulocyte series should be possible with radioautography of marrow and blood samples. With this information, elimina-

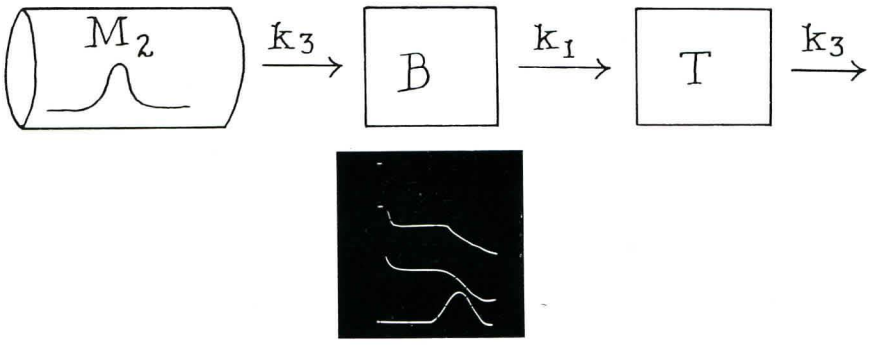

FIG. 7.—A type of granulocyte kinetic model (with type III compartment) with random distribution in the maturation phase. The computor model curve is compared with the in vivo data curve. The standard deviation of random distribution was set at two days.

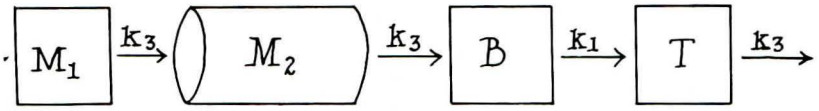

FIG. 8.—The simplest granulocyte kinetic model (model "T") by which the analog computor curve can be made to approximate the in vivo data curve.

1. $-\frac{d\,C_{M_1}}{dt} = \frac{k_3\ C_{M_1}}{V_{M_1}}$

2. C_{M_2} (out) = C_{M_2} (initial) for $t < t_1$

3. $C_{M_2}\ (out)_t = C_{M_2}\ (in)_{(t-t_1)}$

4. $-\frac{d\,C_B}{dt} = \frac{k_3}{V_B}[C_B - C_{M_2}\ (out)]$

tion of several of the present models should be possible, and a closer approximation of the actual granulocyte kinetic model may result.

With granulocyte kinetic models, calculations of certain kinetic data can be made. To demonstrate the type of information that can be gained from analysis of the DFP^{32} curve by means of a kinetic model, the simplest workable model will be used as an example. This model, with its defining formulae, is shown in figure 8. The designation "T" is employed to suggest that, just as with the early Ford cars, when more information about the mechanism is obtained, more and better models will follow.

In the analysis of the DFP^{32} in vivo data curve by this model, phase I represents the removal of blood granulocytes, and because no return of granulocytes from tissues is allowed, this is equal to granulocyte turnover. The granulocyte turnover, in turn, represents production in the steady state. Phase II is the time necessary for transit through the maturation tube. The shape of the curve in phase III is influenced in this model by the rate of granulocyte turnover through blood and the decrease in radioactivity because of cell division in the mitotic

TABLE 2.—*Results of Analysis of Model "T"*

Phase I

$T_{1/2}$ = 6.4 hours
Granulocyte turnover time in blood = 9.4 hours
Number of times the blood granulocyte population is turned over per day = 2.5
Calculated number of blood granulocytes = 25×10^9
Number of granulocytes turned over per day = 62.5×10^9
% of blood granulocytes turned over per hour = 10%

Phase II

Duration = 11 days
Time spent in maturation tube = 11 days
Size of maturation tube = 27.5 × blood granulocytes = 687.5×10^9

Phase III

$T_{1/2}$ = 3 days
Generation time of mitotic pool = 3 days
Size of mitotic pool = 7.5 × blood granulocytes = 187.5×10^9

TABLE 3.—*Comparison of Granulocyte Kinetic Estimates (Expressed as Cells × 10^{-9}/Kg. Body Wt.)*

	Cells Produced Per Day	Total Marrow Population	Mitotic Pool
Osgood		25.7	4.3
Patt	2.6	8.3	1.4
Donohue et al		11.4	2.6
DFP^{32} (Model "T")	0.9	12.5	2.7

pool. Because of the rapidity of cell turnover through blood, the slope of the line is influenced primarily by the effect of cell division in the mitotic pool. Thus, assuming phase III to be a simple exponential decrease, by measuring the half-time, one obtains a fair approximation in this model of the doubling time in the mitotic pool.

With this model, the kinetic calculations presented in table 2 were obtained. Comparisons of these calculations with those of Osgood,[4] Patt,[5] and Donohue et al.[3] are shown in table 3.

Obviously more work is required to define more exactly the granulocyte kinetic model and its relationship to the DFP^{32} data. However, as demonstrated by the analysis of the simple model presented above, one may expect to obtain valuable data concerning granulocyte kinetics in normal and pathologic conditions when these definitions have been established.

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