CURRENT EVIDENCE indicates that isotopic labeling of deoxyribonucleic acid (DNA) results from synthesis during mitotic interphase and that acquired label is retained until cell death and degradation occur. The validity of using inorganic radiophosphorus (P³2) as a leukocyte DNA label rests on these findings.

Leukocyte kinetics have been studied by a number of investigators using DNA-P³2 labeling. Such studies have shown a rise in concentration of label in circulating leukocyte DNA-P for several days after P³2 administration, reaching a peak at a time which varies in different species. Decline in label concentration has been more gradual than the rate of increase. In rabbits, DNA-P specific activity of total leukocytes remains at a low level for at least two days, climbs sharply to reach a peak four days after P³2 administration, and then declines gradually. Lymphocyte DNA-P activity remains at a nearly constant low level during this time, contributing nothing to the general shape of the curve for total leukocytes. Although the peak in circulating leukocyte DNA-P specific activity seems to be produced by events occurring during the first two hours after injection of the isotope, continued labeling of DNA persists for several days after a single intravenous injection of only two microcuries of inorganic P³2/Kg, and contributes significantly to the shape of the curve at least during the period after the peak.

Although nitrogen mustard (HN₂) has generally been considered to act primarily as an inhibitor of cell production, Osgood and Chu showed in cultures of marrow cells that in concentrations of 1:500,000 or greater it regularly killed all granulocytic elements thought capable of mitosis but did not destroy mature granulocytes. This study presents the results of experiments in which leukocyte kinetic alterations were produced by nitrogen mustard given at various times in relation to P³2 administration in efforts to clarify the factors producing the normal curve.

MATERIALS AND METHODS

These studies were done in 3–5 Kg. rabbits, each of which received intravenously 2.0 µc of P³2/Kg. of body weight. Technical and analytical procedures have been given in previous reports. Leukocytes were obtained from heparinized whole blood by the method of Athens et al. Red cells were sedimented in dextran and those remaining in the leukocyte-containing supernate were hemolyzed with gramicidin-lysolecithin. Leukocytes were washed in saline and platelets removed by differential centrifugation.

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remaining leukocyte suspension was fractionated by a modification of the Schmidt-Thannhauser procedure, quantitative phosphorus analysis performed by an adaptation of the method of Berenblum and Chain, and radioactivity determined by scintillation counting. When necessary, blood from several animals was pooled to provide a leukocyte sample adequate for specific activity determination.

Nitrogen mustard (methyl-bis-[beta-chlorethyl]-amine hydrochloride, Squibb Mustargen) was diluted to 1.0 mg./ml. in sterile isotonic saline immediately before use and 2.5 mg./Kg was injected into a marginal ear vein. Leukocyte counts on heparinized heart blood were performed with a Coulter Electronic Cell Counter and hemocytometer counts on ear vein blood were done by standard technics. Counts on ear vein blood, whether done by chamber or the Coulter counter, were routinely higher than counts from heart blood unless a very free flow from the vein was established. Since this was difficult at times, counts on ear vein blood were done on the second drop whenever possible. Differential leukocyte counts were done on smears stained with Wright's stain. Femoral marrow was studied using direct imprints stained with Wright's stain and sections stained with hematoxylin-eosin.

RESULTS

Figure 1 shows typical specific activity curves from normal rabbits of total leukocyte and lymphocyte DNA-P during a nine-day period. Figure 2 presents...
data showing the effect of 2.5 mg. of HN₂/Kg. on total and differential leukocyte counts. Lymphocytes fell precipitously during the first 24 hours to recover partly by the third day, but subsequent recovery did not occur until after the eighth day. During this later period some of the cells counted as lymphocytes were mononuclear cells difficult to classify. In contrast, the level of circulating granulocytes was well maintained after HN₂ for about 2.5 days before falling sharply between days two and three to reach the lowest level on day four. Progressive recovery then took place to reach the preinjection level by day nine. Similar observations were made on eight additional
rabbits at four-hour intervals between days two and three after HN₂ to define more closely the shape and slope of the granulocyte disappearance curve during this interval. The insert in figure 2 shows these data which describe a nonlinear function, possibly exponential, with the onset of rapid decline at 56 hours after HN₂. During the following 16 hours the bulk of the granulocytes disappeared. The absolute lymphocyte and granulocyte counts of animals given 2.5 mg. of HN₂/Kg. are shown in the lower section of figures 3, 4, and 5 for correlation with DNA-P specific activity curves.

HN₂ given immediately and three hours after P³₂ injection produced the effect on DNA labeling shown in figures 3A and 3B. When given immediately after P³₂, no activity was found in circulating leukocyte DNA-P during the
first two days postinjection; a DNA-P specific activity of only 1.25 cpm./μg. was reached on day three. No determination could be made on day four after HN₂ because too few cells were circulating. During the period of granulocyte recovery in the succeeding five days, circulating leukocyte DNA-P specific activity remained relatively constant at about 2.5 cpm./μg. When HN₂ was given three hours after P³² (fig. 3B) this portion of the curve closely resembled that of the preceding group (fig. 3A). No determinations were made in this group before day 5.

The DNA-P specific activity curve of circulating leukocytes of animals treated with HN₂ 24 hours after P³² is shown in figure 4. During the first three days after injection of label, the curve resembles that of untreated animals (fig. 1) with a sharp rise after day two. The portion of the curve after day five is of the same shape and magnitude as found in animals receiving mustard immediately and three hours after P³².

P³² was given to another group of animals four days after HN₂ administration at the time maximal granulocytopenia was present. An entirely different curve of leukocyte DNA-P labeling resulted as shown in figure 5. DNA-P specific activity on day five was 14 cpm./μg., a level much higher than found under any of the previously described circumstances. On subsequent days it
HN2 4 DAYS BEFORE P32

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Fig. 5.—Circulating leukocyte DNA-P specific activity from animals receiving P32 four days after HN2 at time of maximal leukopenia. Number in parentheses indicates number of animals.

fell rapidly but on day eight still remained at a relatively high level. This curve shows a nonlinear decrease in the concentration of DNA-P32 in circulating leukocytes. Femoral marrow from animals three and four days after HN2 administration was found to be virtually aplastic. Recognizable granulocyte precursors were rare.

DISCUSSION
The effect of HN2 on circulating leukocyte counts as reported here is similar to that noted by Graef et al.20 As suggested by Graef, the precipitous fall in lymphocytes during the first day after HN2 likely results primarily from a direct action of the agent on the lymphocyte rather than from decreased pro-
duction. These data, along with the data of others\textsuperscript{5,13,24,25} showing low levels of incorporation of isotopic precursors into lymphocyte DNA, suggest that lymphocyte production as gauged by DNA synthesis is slow relative to granulocyte formation. If this is true, the rapid early lymphopenia after HN\textsubscript{2} likely results largely from increased cell loss. The early partial lymphocyte recovery must be due in part to release of cells which were post-mitotic at time of P\textsuperscript{32} injection since no detectable activity is in circulating leukocyte DNA-P (fig. 3A, day two) when cell numbers are increasing. Some lymphocyte regeneration could contribute to the appearance of DNA label between two and three days when granulocytes are rapidly disappearing and lymphocytes are slowly increasing in number.

Evidence has been presented that there exists a post-mitotic granulocyte reserve of considerable magnitude.\textsuperscript{5,26} DNA labeling studies in normal rabbits\textsuperscript{17} are compatible with this hypothesis and suggest this reservoir is sufficient to replace cells lost from the circulation for at least two days. If the post-mitotic maturation and storage compartment is viewed as a tube connecting the mitotic pool of marrow with the circulation, then cells after final division progress through it in an orderly manner with entry into the circulation a function of time of entry into the tube. A slight degree of random mixing in the post-mitotic compartment would account for the appearance of some labeled elements in the circulation during the first two days after P\textsuperscript{32} injection. The granulocyte disappearance curves (fig. 2) are consistent with a post-mitotic reservoir of this size and would confirm if HN\textsubscript{2} effectively prevents granulocyte production but does not affect the size of the post-mitotic pool or the rate of release of cells from it.

Effective inhibition of granulocyte production by HN\textsubscript{2} is assured for the purpose of this discussion since no cells synthesizing DNA immediately after mustard injection enter the circulation for at least two days (fig. 3A).

The similarity, for the first three days, of the curve of leukocyte DNA-P specific activity of animals given HN\textsubscript{2} 24 hours after P\textsuperscript{32} (fig. 4) to that of control animals (fig. 1) suggests that the dose of mustard used does not affect the size of the post-mitotic reservoir or alter the rate of release of cells from it. It is obvious on comparing figures 3A and 4 that by 24 hours a significant number of granulocytes labeled and entered the post-mitotic reservoir. If HN\textsubscript{2} increased the rate or altered the manner of release of cells from the reservoir, these labeled elements should not have appeared in the circulation on schedule as they seem to have done. One other possible circumstance would yield the same results: a simultaneous decrease in reservoir size, rate of release from the reservoir, and rate of loss from blood. It would be an amazing coincidence if these occurred concomitantly so as to give data indicating a reservoir size equal to that as determined in normal animals by other means.

Based on the above, limits to circulating granulocyte half time (T\textsubscript{1/2}) may be established from the disappearance curve (fig. 2), assuming the onset of rapid decline marks depletion of the post-mitotic pool. Rate of fall between 56 and 60 hours defines a T\textsubscript{1/2} of about 2 hours while that between 60 and 96 hours indicates a T\textsubscript{1/2} of about 7 hours. In other studies in normal rabbits with in vivo diisopropylfluorophosphate (P\textsuperscript{32}) labeling,\textsuperscript{27} data were obtained which
were consistent with a T½ of 2–2.5 hours. The longer half-time derived from decrease in granulocyte concentration between 60 and 96 hours might be due to release of some newly formed elements during this interval.

A post-mitotic granulocyte reservoir about 40 times the number circulating would provide sufficient cells to satisfy a T½ of 2 hours if, as has been suggested, loss from the blood is a random process, and there exists an intravascular marginated pool of equal size to and in rapid equilibrium with the circulating pool. This number of granulocytes should represent the total body store of extravascular post-mitotic granulocytes which can enter (or re-enter) the circulation.

Lack of precise information about DNA labeling time and the characteristics of labeling has been a major obstacle to translating DNA specific activity curves into biologic terms. DNA labeling after one injection of P³² has been shown to continue at changing rates for a considerable period. It has been suggested that there is in normals receiving a single injection of P³² an exponential decrease in the amount of label per cell as proliferation continues. The studies reported here indicate that the availability of labeled precursor phosphate relative to onset and duration of DNA synthesis is the fundamental determinant of the labeling characteristics of newly formed cells. After the time of maximal leukopenia in groups given HN₂ immediately, 3 hours and 24 hours after P³², the DNA-P specific activity curves are nearly identical (figs. 3A, 3B and 4). Leukocyte kinetics are comparable in each group during this interval; rapid granulocyte recovery is occurring from 5 to 10 days after P³² administration when distribution of labeled phosphate has been accomplished and rate of loss from the body is quite slow. It is concluded that the newly formed cells entering the circulation under these conditions must bear on the average the same concentration of DNA label per cell to provide the flat specific activity curves obtained.

On the other hand, the administration of P³² at the time of maximal leukopenia, to animals having leukocyte kinetics comparable to the above groups, resulted in the curve shown in figure 5. This curve is explained most reasonably by the production and release of cells bearing a decreasing amount of DNA label per cell. The only apparent difference between this and the other groups is the availability of labeled precursor phosphate. Thus, although the suggestion may be true that in normal animals there is an exponential decrease in label with cell division, definitive evidence is not yet available.

**SUMMARY**

The effect of a large dose of nitrogen mustard on total and differential leukocyte counts in rabbits has been studied in conjunction with the effect on DNA labeling with inorganic radiophosphorus. Considered together, the findings indicate that this dose of nitrogen mustard has a direct lethal effect on lymphocytes and effectively blocks P³² incorporation into leukocyte DNA for several days by killing the elements capable of division. The post-mitotic granulocyte reservoir seems to remain intact and is of sufficient size to replace those lost from the circulation for about 56 hours. Granulocyte circulating half-time under the conditions of these experiments falls between two and
seven hours. The post-mitotic granulocyte reservoir is calculated to be 40 times the size of the circulating pool. During the period of rapid granulocyte recovery, leukocytes have been shown to exhibit a decreasing concentration of DNA label per element and maintenance of the same level of label per element depending on the availability of precursor $^{32}$P. At present no conclusion about DNA labeling characteristics with inorganic $^{32}$P under steady state conditions is warranted.

**Summario in Interlingua**

Le effecto de un grande dose de mustarda a nitrogeno super le numeracion total e differential de leucocytes in conilios eseva studiate in conjunction con le effecto del marcation de acido disoxyribonucleic (ADN) con radiophosphoro. Le resultatos, considerate conjunctemente, indica que iste dose de mustarda a nitrogeno ha un directe effecto letal super lymphocytes e efficacemente bloca le incorporation de $^{32}$P ad in ADN leucocytic durante pluri dies per occider le elementos capace de division. Le reservoir de granulocytes post-mitotic apparentemente remane intacte e su grandor es sufficiente pro reimplaciar granulocytes que es perdite ab le circulasion durante approximativamente 56 horas. Sub le conditiones de iste experimentos le tempore de medie valor de circulante granulocytes es inter duo e septe horas. Il es calculate que le reservoir de granulocytes post-mitetic es 40 vices plus grande que le massa de granulocytes circulante. Durante le periodo de un rapide restablimento de granulocytes, le leucocytes exhibiva un decrescente concentration de marcare de ADN per elemento e le mantenentia del mesme nivello de marcare per elemento dependente del disponibilitate de $^{32}$P precursori. Al presente nulle conclusion es justificate in re le caracteristicas del marcage de ADN con inorganic $^{32}$P sub conditiones de stato constante.

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Leukocyte Kinetics Studied by Nitrogen Mustard Induced Alterations in DNA Phosphorus Labeling

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