Erythropoietin: Hypothesis of Action Tested by Analog Computer

Abstract. *The action of erythropoietin on the pool of undifferentiated bone marrow cells has been examined with the aid of a model tested with an analog computer. The model is consistent with reported experimental results. The essential aspects of the action are (i) the effect is exerted during the S phase of the cell's cycle; (ii) "effective" erythropoietin is present in the cell only during G_1 and part of S; and, (iii) hormone molecules survive in the cell for only a limited time in effective form and require a certain time to assume this form.*

The bone marrow and spleen of mice made polycythemic by hypertransfusion contain no morphologically identifiable nucleated precursors of red blood cells. If erythropoietin—a substance that can be obtained from blood plasma of anemic animals (*1*)—is injected into such hypertransfused mice, there follows an orderly appearance of dividing and maturing erythrocyte precursor cells (*2*). These were known to morphologists and had been classified before erythropoietin was discovered. The cellular changes in the hemopoietic organs are followed in several days by the appearance of reticulocytes in the circulating blood (*3*). For these and other (*4*) reasons, the chief, but perhaps not the only (*5*), function of erythropoietin is believed to be that it causes cells of a primitive and biochemically undifferentiated type to undergo the differentiation necessary for hemoglobin synthesis and morphological evolution to the mature circulating red blood cell. It is assumed, in the following discussion, that biochemical and morphological differentiation is ultimately determined by information contained in DNA molecules in the primitive and undifferentiated cells of the hemopoietic organs and that erythropoietin "derepresses" this information (*6*) by a mechanism similar to the derepression of protein synthesis in bacterial systems as proposed by Jacob and Monod (*7*). That is, erythropoietin is taken to be an inducing metabolite which reacts with the repressor product of the regulator gene or genes that control the genetic operons controlling hemoglobin synthesis and cytodifferentiation. From this basic and speculative subcellular view of how a hormonal substance can set in motion a controlled and complex sequence of biochemical and morphological events, I now consider the mode of action of erythropoietin at the cellular level.

When mice are exposed to 900 roentgens total-body x-radiation they die in about 10 days from a profound hemopoietic aplasia. If, however, they are injected with as few as 10^6 bone-marrow cells taken from unirradiated mice of the same strain, 75 percent or more will live, and the number of bone-marrow cells in their femurs (*8*) will return nearly to normal by 15 days. Most of the marrow cells injected are differentiated hemic cells of the granulocytic, erythrocytic, and megakaryocytic lines and of lymphoid cells. The differentiated cells are capable of only a limited number of divisions (3 to 5 for the most primitive). Since the regenerated hemopoietic system of the recovered mice contains 1000 or more times the number of cells injected, there must be a fraction of the injected bone-marrow cells which is capable of unlimited division and which can also give rise to the differentiated and mature cells that leave the hemopoietic organs and enter the circulation. Cells with such capabilities are generally referred to as stem cells, but their morphological characteristics and homogeneity are controversial. There is autoradiographic evidence, however, that in mice (*9*) the stem cell of the bone marrow is the "small round cell" and that this cell can give rise to all the differentiated hemic cell lines. It is generally agreed that stem cells are either themselves sensitive to erythropoietin or give rise to a subclass of "erythropoietin-sensitive" but undifferentiated cells. The question of homogeneity of the undifferentiated cell pool is not relevant here except for the assumption that, if the "erythropoietin-sensitive" cell is a subclass of the hemic undifferentiated cell population, it nevertheless is of stem type II (*10*). This stem type is characterized by a feedback control that can prevent cells from entering into cell cycle. Thus, rate of division of cells in this population will decrease as the population grows until the addition of new cells to the population just balances the loss of cells from the population and a controlled, steady population size can result.

This report is based on a computer analysis of a model of erythropoiesis developed (*11*) from experiments in which mice are given lethal total-body x-irradiation and treated with normal bone-marrow cells. The model is of interest in connection with the study of the kinetics of recovery of the hemopoietic system in these treated mice (*12*). The aspects of the model that are relevant to this discussion are (i) the undifferentiated cell pool is assumed to be homogeneous with respect to probability of division and erythropoietin sensitivity—that is, there are no subclasses in the undifferentiated cell pool; (ii) the cells are assumed to go through a cell cycle (*13*) made up of a postmitotic interval (G_1), followed by an interval (S) of DNA synthesis requiring about 7 hours to complete, then by a premitotic interval (G_2) of 1 hour, and finally mitosis (M), requiring ½ hour; and (iii) the feedback control that makes this a population of stem cells of type II is assumed to operate by controlling the probability that cells in G_1 will enter S. Thus, the average length of G_1 will vary according to the size of the population. In this report, G_1 is assumed to vary between a minimum of a few hours and a maximum of a few days (*14*), depending on the rate at which cells are leaving the undifferentiated cell pool because of differentiation or death.

These assumptions can be used (*15*) to write a system of differential equations that describe the number of cells in G_1, S, G_2, M and the sum of all these to give the total undifferentiated

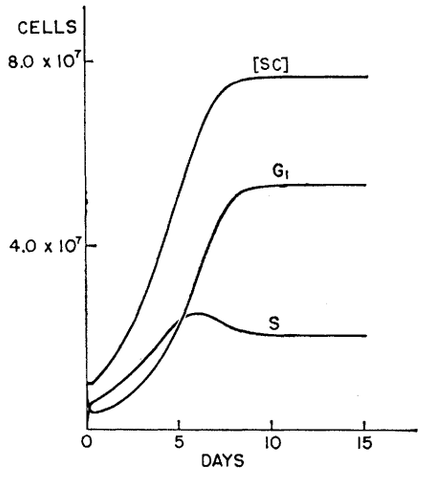


Fig. 1. Analog computer output of model described. Curve [SC] traces the increase in the number of undifferentiated cells. G_1 and S shows the number of these cells in the G_1 and S phase of cell cycle, respectively. To maintain graphic simplicity, the number of cells in G_2 and M are not shown.

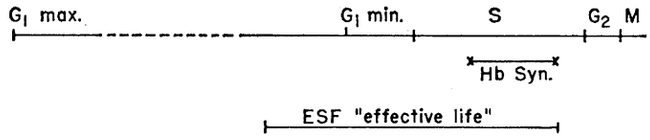
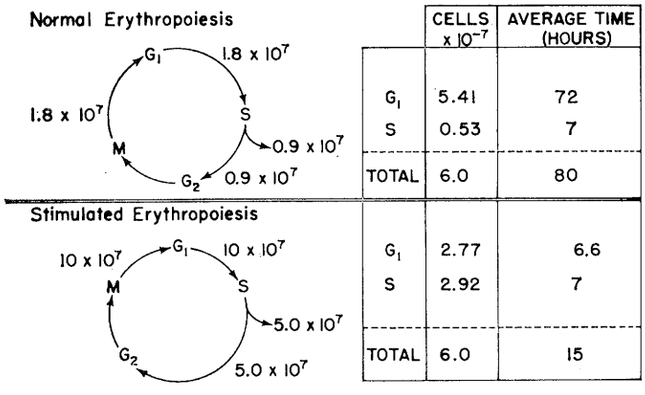


Fig. 2. Hypothesis of action of erythropoietin. The length of G_1 is assumed to vary between some maximum, G_1max, and minimum, G_1min, limit. The genes relevant to hemoglobin synthesis and cytodifferentiation are assumed to be replicated during a certain interval in S, hemoglobin synthesis (Hb syn.). The possible effective life of erythropoietin (ESF) is assumed to occupy part of G_1 and S but is longer than G_1min. Effective hormone, however, is assumed to be necessary during hemoglobin synthesis for the cell to be triggered into erythroid differentiation.



	CELLS x 10^{-7}	AVERAGE TIME (HOURS)
G_1	5.41	72
S	0.53	7
TOTAL	6.0	80
G_1	2.77	6.6
S	2.92	7
TOTAL	6.0	15

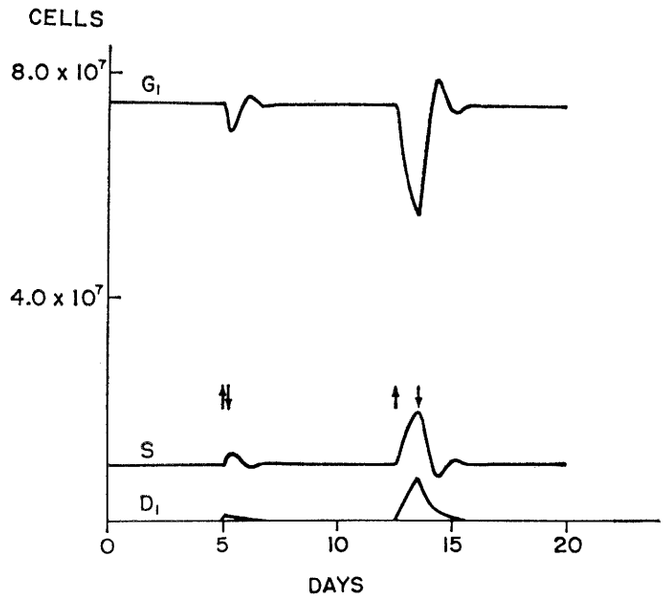


Fig. 3. Analog computer simulation of the action of erythropoietin on the undifferentiated cell population (see text); G_1 and S as in Fig. 1, D_1 traces the number of cells in the earliest erythroid pool (pronormoblast).

Fig. 4 (left). Shows the undifferentiated cell pool at two levels of erythropoiesis. The numbers on the arrows indicate the flux of cells. The number of cells in G_2 and M is 1/14 and 1/28, respectively, of the number in S since only the length of G_1 is assumed to vary.

cell pool (SC). From the assumed average time in each phase, parameters for the flux rate from S to G_2, G_2 to M, and M to G_1 are calculated. The flux rate of cells from G_1 to S is made dependent on size of the undifferentiated cell pool. In the analysis of erythropoietin action, the fractional loss of S cells to differentiated cell pools is assumed to be proportional to erythropoietin concentration. Electronic components in the computer, programmed according to the model, become analogs of the biological system; for example, the voltage stored in an integrator represents the number of cells in G_2, and the setting of a potentiometer represents the flux rate of cells from G_2 to M. The use of the analog computer to solve the set of differential equations that constitutes the model eliminates long, tedious, or occasionally impossible, analytical solutions (*16*).

Figure 1 is an analog computer solution for a recovering undifferentiated cell population (SC), read out on an X-Y plotter. The initial condition (zero time) was that the population consisted of 10^7 cells all in G_1. Since this is only about ⅛ the steady-state population size, cells rapidly enter S and the population (SC) begins to grow. For the first few days proliferation is unrestricted, G_1 is only about 3 hours, and the total generation time is 11 to 12 hours. As the population passes the half-maximum size at about 5 days the feedback control comes increasingly into play, and the probability that G_1 cells will enter S progressively decreases with a consequent progressive increase in the average generation time. Finally, at 8 to 9 days most of the undifferentiated cell population (SC) is in G_1, and the number of cells passing through S, G_2, and M is only enough to balance the loss due to differentiation; the population is stable at approximately 8×10^7 cells. This population is continuously delivering 1.8×10^7 cells per day, after day 10, to the erythroid series, thus maintaining a daily production of 0.29×10^9 new red blood cells which is the estimated requirement for a mouse with 1.6 ml of total blood volume (*12*). Cells are also being delivered to maintain other differentiated hemic cell lines and to replace a small fractional loss of undifferentiated cells to the circulation (*17*) and from accidental death in the hemopoietic organs. The total flux of cells through S is about 6 to 7×10^7 cells per day, and the average time in G_1 is about 22 hours, giving a generation time of about 31 hours (*18*).

How could erythropoietin act on an undifferentiated cell population of this type to control the rate of erythropoiesis? Figure 2 is a diagrammatic statement of a mode of action of erythropoietin that is consistent with the observations and assumptions outlined above. Erthropoietin is assumed to be an "inducing metabolite" in the sense of the Jacob and Monod hypothesis (*7*) and to act during the replication (*19*) of that part of the genome that controls the synthesis of the enzymes necessary for hemoglobin synthesis and cytodifferentiation. It is assumed, therefore, that hormone must enter the cell before the replication of the relevant gene or genes in S. Further, after penetrating the cell, a certain time is assumed necessary before the hormone becomes "effective" and also that this "effective form" can be catabolized so that erythropoietin has only a restricted "effective life" within an undifferentiated cell. Thus, a cell will be derepressed only during the effective life of erythropoietin, and this must continue into S. The fraction of cells that are derepressed in a given undifferentiated cell population will depend upon

the length of G_1 as well as on the level of erythropoietin. But a derepressed cell (that is, a cell with an "effective load" of erythropoietin) is not necessarily "triggered" since, if G_1 is prolonged beyond the effective life of the hormone, some cells will become repressed again before entering S.

In the recovering population (Fig. 1), during the first few days only a relatively small fraction of the population is in G_1, and G_1 is short; the population as a whole is therefore resistant to erythropoietin as compared to a later time when over half the population is in G_1. On the other hand, after 10 days G_1 is prolonged, and the population as a whole is again relatively resistant to erythropoietin. It is only during the intermediate time of recovery, 5 to 7 days, that the population as a whole shows maximum sensitivity to the erythropoietin. Maximum sensitivity will prevail in a population when the length of G_1 approximates the effective life of the hormone in the cells.

The essential features of this mode of action of erythropoietin, therefore, are (i) the effect is exerted during S; (ii) effective erythropoietin is present in the cell only during G_1 and part of S; and (iii) hormone molecules survive in the cell for only a limited time in effective form and require a certain time to assume this form.

Figure 3 is output of an analog computer experiment recorded on an X-Y plotter as in Fig. 1. The differentiation of cells into erythroid elements is assumed to be zero, as in a hypertransfused animal, and the fraction of the population in S is, therefore, less than in the recovered population (day 10 to 15) of Fig. 1. The fraction of the population in G_1 is greater, and the average time in G_1 is about 53 hours. The average generation time in this population is about 62 hours.

The action of erythropoietin was simulated by "triggering" a constant fraction of the cells in S into the earliest erythroid pool (D_1). The time during which this constant was applied was controlled by a function switch in the computer. At the first upward-going arrow in Fig. 3, cells from S begin to enter the earliest erythroid pool D_1. These cells, instead of dividing and entering G_1, are lost to the undifferentiated cell pool which, therefore, begins to decrease in size. The size decrease releases the feedback inhibition holding cells out of S, and the number of cells in S begins rapidly to increase. However, erythropoietin is allowed to act for only a few hours (as indicated by the downward-going arrow) (*20*), and most of the cells brought into S stay in the undifferentiated pool; very few cells enter the erythroid line, and the whole population returns rapidly to equilibrium.

On the other hand, if erythropoietin is allowed to act for 24 hours (the second upward-going arrow, Fig. 3) more and more G_1 cells are brought into S, and G_1 is progressively shortened so that the population becomes increasingly sensitive to erythropoietin. Because of this increasing sensitivity the early erythroid pool increases rapidly during the time of action of the erythropoietin. As this action continues, the fraction of the population in G_1 decreases, and G_1 becomes progressively shorter until finally it would be shorter than the effective life of erythropoietin, and the population would become resistant to the action of the hormone. This prediction of the model, that the undifferentiated cell pool in a hypertransfused animal becomes progressively more sensitive to erythropoietin, is supported by Schooley's experiments in mice (*21*). He showed that the response to erythropoietin increased by a factor of about 2.5 to 3.0 at 24 to 36 hours after initial stimulus with hormone and then returned to or below the response observed without prior stimulus. The decreasing sensitivity 36 to 48 hours after initial stimulus was presumably due to decreasing effects of the initial stimulus. The results suggested that erythropoietin caused "recruitment" of undifferentiated cells into a subclass of highly sensitive cells.

In terms of the recruitment hypothesis, the model suggests that erythropoietin acts in a hypertransfused mouse to adjust the undifferentiated cell pool to maximum sensitivity by increasing the fraction of the population that is going into S and by shortening G_1 so that an effective load of erythropoietin will not have a chance to decay before the cell enters S. This effect on the population will be self-limiting since if a high degree of stimulation continues, G^1 would become too short and the fraction of the population in G_1 too small for sensitivity to erythropoietin. It would be impossible to deplete the undifferentiated cell pool with even massive amounts of erythropoietin, and there would be a certain maximum response to increasing amounts of erythropoietin which, once reached, would not change with further increase in hormone. This limit in response to erythropoietin is characteristic of the response of plethoric mice to increasing doses of hormone (*22*), and erythropoietic response in mice recovering from lethal irradiation after treatment with isologous bone-marrow cells is not influenced by exogenous erythropoietin (*23*).

It can also be shown that the (SC) population of the model can support a greatly increased rate of erythropoiesis without a reduction in size of the total population. Figure 4 shows how a population of 6.0×10^7 undifferentiated cells can deliver from 0.9×10^7 to 5.0×10^7 cells per day for erythroid differentiation. The increase is possible since the average generation time of the cells is flexible because it is assumed that G_1 can vary. It is implicit in the model, however, that there be a temporary reduction in the size of the (SC) population to release it from feedback control of proliferation. A temporary reduction in erythropoietic capacity of spleen cells, taken from bled donors and injected into lethally irradiated mice, has in fact been observed (*24*).

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$$(SC) = G_1 + S + G_2 + M$$

where (SC) is the number of all the undifferentiated cells; and, G_1, S, G_2, and M are the numbers of cells in each of these phases of the cell cycle;

$$dG_1/dt = -[A - a(SC)]G_1 - bG_1 + 2cM$$

where a is a constant that represents the rate of decrease, with increasing $[SC]$, in the probability that a cell can leave G; A is a constant computed from the minimum average time that a cell is assumed to spend in G_1; b is a constant representing fractional loss of undifferentiated cells to the circulation and loss by accidental death in the narrow; c is a constant computed from the average time a cell is assumed to spend in mitosis (M);

$$dS/dt = [A - a(SC)], - eS$$

where e is a constant computed from the average time a cell is assumed to spend in S;

$$dG_2/dt = [eS - (f + g)eS] - hG_2$$

where f and g are the fraction of cells, leaving S, that are triggered into erythroid or nonerythroid differentiation, respectively; h is a constant computed from the average time a cell is assumed to spend in G_2; and

$$dM/dt = hG_2 - cM$$

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Phloem Differentiation: Induced Stimulation by Gibberellic Acid

Abstract. *Gibberellic acid solutions supplied through micropipettes to explants from dormant branches of white pine* (Pinus strobus *L.*) *stimulate changes in the cambial zone. Immature sieve cells expand radially and exhibit cytological changes usually associated with spring maturation. Differentiation of sieve cells continues in response to treatment and is recognized by the birefringence of secondary walls when examined with the polarizing microscope.*

Evidence accumulated in recent years suggests that the induction and differentiation of vascular tissues in plants is regulated by the participation of endogenous growth regulators. Experimental findings from the laboratories of Jacobs (*1*), Torrey (*2*), and Wetmore (*3*) provide strong support for the involvement of auxin, indole-3-acetic acid (IAA), in various stages of xylem regeneration and differentiation. While IAA is generally recognized as a limiting factor for xylem differentiation, other growth substances (*4*), sugars (*5*), and minerals (*6*) also may be involved.

Physiological studies on the role of growth substances in regulating phloem differentiation are limited. LaMotte and Jacobs (*7*) view phloem regeneration in wounded *Coleus* plants as proceeding basipetally, stimulated by the availability of endogenous IAA flowing towards the base from shoot organs. Indole-acetic acid appears, then, to be a limiting factor for phloem regeneration, although other factors could be limiting when IAA is present in adequate amounts. Sucrose, alone or in combination with IAA, was not effective in inducing phloem differentiation in their system (it was probably never limiting in their green *Coleus* plants which were illuminated 16 hours per day) but has been implicated as a major factor in another experimental system (*3*).

Gibberellic acid has been noted to stimulate the differentiation of secondary phloem in woody species (*8*), and the stimulation is enhanced when this acid and IAA are supplied in combination. However, Wareing *et al.* (*9*) have been unable to detect stimulated differentiation of sieve elements with gibberellic acid treatment.

An evaluation of existing data leads to the consideration that phloem differentiation—like xylem differentiation—is controlled by a variety of factors, one or more of which may become limiting at some stage during development from cell initiation to maturation. Evidence presented in this report establishes a functional role for gibberellic acid during sieve cell differentiation in explants of white pine (*Pinus strobus* L.). To evaluate the activity of the acid in phloem differentiation, explants from dormant branches of white pine were utilized. Transections of these branches are characterized by a cambial zone of six to ten rectangular cells with thickened tangential walls, limited internally by latewood tracheids and externally by sieve cells in various stages of maturation (Fig. 1*A*). Except for the cambial initials, certain cells of the cambial zone, even at this stage, are already destined to become phloem cells. Abbe and Crafts (*10*) demonstrated that the initial response to growing conditions in the spring is seen in the swelling of cells in the cambial zone. Associated with the swelling are thinning of the tangential walls, cytoplasmic changes, loss of the nucleus, and deposition of secondary wall—a sequence of changes depicting sieve cell differentiation. These anatomical changes, easily seen by microscopic techniques, provide a convenient assay for detecting substances capable of stimulating phloem differentiation.

Explants were excised from surface-sterilized pine branches (Fig. 2*A*), and sterilized micropipettes (*11*) were inserted into the soft tissue of the cambial zone. Explants with pipettes were immediately planted upright in sterile test tubes containing agar-solidified Knudson's medium with 1 percent sucrose (Fig. 2*B*). Sterilized solutions of gibberellic acid to be tested for their ability to induce differentiation of sieve cells were supplied to the cambial zone via the micropipettes. Tubes were sealed with polyethylene and maintained in a temperature-controlled room (25° ± 2°C). They received 12 hours of illumination daily (about 2200 lu/m^2) supplied by a combination of fluorescent and incandescent lamps. Solutions were maintained at a constant level and all experimental treatments were prepared in triplicate on three separate occasions during February. Controls consisted of explants supplied with empty pipettes. Explants were collected at weekly intervals for a 3-week period, processed for microscopic examination, and studied by light and polarized light microscopy.

Control explants viewed with polarized light (Fig. 1*B*) exhibit the usual features associated with the dormant condition. The over-wintering cambial zone and immature sieve cells are nonbirefringent, as are the large, banded parenchyma cells which delimit seasonal increments of phloem differentiation and help form detectable "growth rings." Situated between these two nonbirefringent zones, and in striking con-