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Erythropoietin, Its Nature and Mechanism of Action

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Introduction.

The hormone erythropoietin is a substance that regulates the formation of red cells in the hemopoietic tissues of the body. It has been shown that under conditions of anoxic stress due to anemia, low atmospheric O₂ tension or any other cause an erythropoietic factor is demonstrable in the plasma of animals. Carnot and Deflandre⁽¹⁾ made the first claim to this effect as long ago as 1906. It was only relatively recently however that several groups of investigators presented evidence supporting the hypothesis that erythropoietin is part of the regulatory system for red cell formation in the normal organism. For instance the presence of small amounts of erythropoietin can now be demonstrated in both the plasma^(2, 3) and urine^(4, 5, 6) of normal unstressed animals and human beings. Moreover, antibodies to erythropoietin injected into normal animals cause them to become anemic⁽⁷⁾.

The following regulatory circuit seems to exist⁽⁸⁾. When the oxygen tension in the body falls below a critical level a sensor signals to the sites of production of the hormone in the kidney and perhaps elsewhere. Erythropoietin appears in the circulation and interacts with susceptible multipotent stem cells in the bone marrow and other sites of erythropoiesis causing them to start development in the red cell line. After several divisions have occurred the progeny of these cells appears in the blood stream. The additional oxygen carried by the new cells leads to a higher oxygen tension in the tissues and the output of erythropoietin is reduced accordingly.

Chemical Nature, Purification.

The chemical nature of the hormone is still largely unknown. This is mainly because only extremely small amounts of it are available in a highly purified form. From an anemic plasma with about 0.007 units per mg protein to the most highly purified fraction with 6500 units per mg protein there is approximately a million-fold increase in potency but unfortunately only a 2 percent yield. This purification was achieved by Goldwasser and Kung⁽⁹⁾ by a procedure involving adsorption of dialyzed plasma on DEAE, chromatography of the active eluate on amberlite XE-97, ammonium sulfate precipitation, chromatography of the active fraction on sulfoethyl sephadex gel followed by calcium phosphate gel adsorption and fractional elution (Table 1). Lowy and Keighley⁽¹⁰⁾ have reported a con-

siderable purification of erythropoietin from human urine, and Contrera, Gordon and Weintraub⁽¹¹⁾ have described purification of a renal erythropoietic factor.

The hormone is generally assumed to be a glycoprotein. The information on the chemistry of erythropoietin collected by various groups is based mainly on inactivation studies done on very impure preparations. These studies suggest that the erythropoietin molecule contains a polypeptide. It loses its biological activity on treatment with trypsin implying the presence of lysine and/or arginine in the peptide⁽¹²⁾. Fluorescein isothiocyanate, which substitutes on phenolic hydroxyl groups and on free amino groups, inactivates erythropoietin. This study by Lowy and collaborators⁽¹³⁾ suggests a possible involvement of tyrosine, lysine, or an N-terminal amino acid in a site required for activity. These workers have also shown, using anhydrous formic acid which can react with serine or threonine hydroxyl groups, that these are not involved in the hormone's activity. Sialic acid may also be a part of the erythropoietin molecule. Its removal from preparations, either with dilute acid⁽¹⁴⁾ or sialidase⁽¹⁵⁾, led to a total loss of activity in the in vivo - animal assay, but not in the in vitro assays⁽³⁾.

Assays.

The in vivo assays are based on the measurement of the increased incorporation of radioactive iron into red cells of rats or mice treated with the hormone. Usually the endogenous erythropoietin production in these animals is reduced by starvation⁽⁸⁾, polycythemia⁽²⁾, or some other means, prior to the assay.

The in vitro systems are based on the incorporation of radioactive precursors into cells in primary explant culture usually from rat bone marrow. One such system involves the increased incorporation of ⁵⁹Fe into heme of hemoglobin of cells when erythropoietin is added in vitro⁽¹⁶⁾. I shall refer to this assay system several times later on. The other system involves the stimulated incorporation ¹⁴C-glucosamine into bone marrow cells⁽¹⁷⁾.

The unit of hormone is presently based on an international standard preparation. This in turn was originally standardized in the in vivo-⁵⁹Fe rat system which equated one unit to the response obtained when 5 μ Moles of CoCl₂ were injected into the assay rat⁽¹⁸⁾.

The Glucosamine Incorporation System.

In the following I shall mainly deal with the glucosamine incorporation system since my own work is principally concerned with it. The studies to be presented are based on collaborative efforts with Dr. Goldwasser, and at various times with Drs. Takaku and Gallien-Lartigue. The basic observation was that the addition of erythropoietin caused increased incorporation of ^{14}C -glucosamine into the acid insoluble material of bone marrow cells⁽¹⁹⁾. Most of the earlier data are based on the use of 50% medium 199-50% calf serum and incubation in tubes gassed with 95% air-5% CO_2 . More recently standard conditions have been modified: cells are incubated in plastic culture dishes in 50% medium NCTC 109-50% calf serum. We also use Millipore filters to collect, wash, and when needed acid precipitate cells on them⁽²⁰⁾. It is interesting that the system works best in the presence of calf serum and not with the homologous rat plasma. Fetal calf serum, which is optimal for ^{59}Fe incorporation into heme, seems to be inhibitory for glucosamine incorporation.

A. Specificity.

The specificity of the erythropoietin effect in vitro was tested with respect to cells affected.

Rat bone marrow cells showed an increased incorporation of glucosamine after the second hour of culture in the presence of erythropoietin. The hormone caused equal increments of glucosamine incorporation over the control incorporation per unit time^(17,19). I shall return to the time course of the hormone effect later.

Mouse spleen cells responded to erythropoietin in essentially the same way as rat marrow cells. This was to be expected since in the mouse the spleen is a hemopoietic organ. Both normal and polycythemic mouse spleen cells, the latter not containing any recognizable red cell precursors, were tested⁽²¹⁾.

Rat reticulocytes incubated in vitro did not respond to erythropoietin. This was tested both with the incorporation of glycine into heme and of glucosamine into various unidentified cellular constituents⁽³⁾. Peripheral red cells from rats incorporated glucosamine at a level at most 1-2% that of marrow cells, and did not respond to erythropoietin⁽²¹⁾.

Granulocytes from a peritoneal exudate of rats were obtained by the

method of Gordon⁽²²⁾. They showed very active incorporation of glucosamine when they were incubated the same way as bone marrow cells. The addition of erythropoietin, however, did not cause any increase of this incorporation over a 24 hour period⁽²¹⁾.

These data on cell specificity are all compatible with the hypothesis that erythropoietin acts on hemopoietic stem cells.

The specificity of the effector, that is, erythropoietin itself, in bringing about the increased glucosamine incorporation was tested. This was done by adding erythropoietin at various stages of purification to the marrow cell incubation system. Anemic plasma, (the starting material), and various purified fractions were all active in stimulating glucosamine incorporation in vitro with a fair correlation to their potency as established by the in vivo erythropoietin assay⁽¹⁷⁾. Step III erythropoietin was the hormone standard preparation used in the experiments reported here. The activity in the glucosamine incorporation system of the highest purified fractions was about 30-40% of the activity predicted for them on the basis of the in vivo assay. This allows one to state that it is very likely that erythropoietin and the factor responsible for increased glucosamine incorporation are one and the same. If this were not so they would have had to be present in a relatively constant ratio in fractions representing a 10,000 fold range of potency.

B. Glucosamine Labeling.

I would like to turn now to what little is known about what the ¹⁴C-glucosamine is incorporated into in the bone marrow cells. On the average about 55% (range 35-75%) of the total radioactivity in the cell is found in the acid insoluble fraction. Water lysis of the cells followed by centrifugation yields a similar fraction with about 60-70% insoluble radioactivity. For simplicity we have usually used the acid precipitation procedure but we have referred to both fractions as stroma since the acid precipitable fraction certainly includes the water insoluble cell constituents. During the first 24 hours of incubation the action of the hormone is similarly reflected in the radioactivity of both the soluble and stroma fractions.

The stroma fraction was investigated further as follows⁽¹⁷⁾: Stroma from cells cultured for 24 hours with and without erythropoietin was obtained by the acid procedure. Samples were hydrolyzed for 2 hours at 80° with 0.05 N H₂SO₄ according to the procedure of Gottschalk⁽²³⁾

which releases neuraminic acids. Both the control and the stimulated sample had approximately 56% of their radioactivity solubilized by this procedure. After the removal of H_2SO_4 with the liquid anion exchanger LA-2 the soluble fractions were chromatographed on paper. The distribution of labeled material was similar in the chromatograms of both control and stimulated sample. Neuraminic acids accounted for about 50% of the total radioactivity applied to the paper, 40% remained at the origin. This allows one to postulate that at least 30% of the total stroma radioactivity resides in neuraminic acids. More work is in progress at this time on the fate of the glucosamine label in the cells.

C. Dose Response.

When the relationship between amount of erythropoietin and response in terms of increased incorporation of glucosamine is plotted on arithmetic coordinates it yields an S-shaped curve typical for hormone effects. The same kind of curve is obtained also when the data from the in vivo assay are plotted similarly⁽²⁴⁾. More often however the response is expressed on a linear scale as percent increase of specific activity of the acid insoluble residue over that of the control while the dose of hormone is plotted on a logarithmic scale. This method yields a straight line relationship over part of the response range useful for assaying purposes. Different culture conditions gave different dose ranges within which this "quasi linear" response was obtained⁽¹⁷⁾. (See also Fig. 2). It should be pointed out that this assay must be used very cautiously because of the presence of an inhibitor in the erythropoietin preparations. This causes a depression of glucosamine uptake and affects the range of response. The inhibitor seems to be largely removed from the 0.67 saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate (Step IV), it is therefore concentrated in the corresponding supernatant fraction⁽¹⁷⁾.

When in the ^{59}Fe in vitro system⁽¹⁸⁾ the ^{59}Fe uptake into heme is plotted against the log of the dose, the resulting dose response curve is very similar to the one just described for glucosamine incorporation.

For the experiment described in Fig. 1 the modification of the glucosamine-system utilizing incubation in plastic culture dishes, which has already been mentioned, was used. The presentation chosen here and generally in our more recent work is a plot of % increase in incorporation on a log-scale against dose on a log-scale. This log-log presentation was adopted because it yields a more useful

quasi linear range at low doses than the semilogarithmic plot. For the experiments* used in these curves cells received erythropoietin at the start and label at an interval of from 18 to 21¹/₂ hours of incubation. Both total cells washed and collected on Millipore filters, and their acid insoluble residue prepared on the filter, gave essentially parallel dose response curves, reflecting a similar erythropoietin effect on several classes of labeled compounds in the cell. In the control cells the relative proportion of acid insoluble radioactivity was slightly higher than in erythropoietin treated cells. This causes the percent increase curve for the acid insoluble fraction to be displaced below that of the total sample.

The glucosamine incorporation assay compares favorably with respect to sensitivity with the in vivo assay involving ⁵⁹Fe in vitro system⁽¹⁶⁾. They can all detect reliably about 0.05 units of hormone. The upper limit of usefulness of the glucosamine incorporation assay is set by the number of responding cells in the cultures, the culture conditions in general, and the amount, if any, of inhibitory material present in the sample to be assayed.

From the minimal detectable dose in vitro one can, by making some rather uncertain assumptions, obtain the number of hormone molecules per cell needed for action. Assuming a molecular weight of 50,000 for erythropoietin, a potency of the pure hormone of 10,000 u/mg and a maximum of 1% of the cells in the culture actually responding, one obtains as an order of magnitude estimate about 10⁵ to 10⁶ molecules required per cell. That is a large number of molecules, suggesting that perhaps only a few of them actually penetrate into the interior of a cell. This is at any rate compatible with the observation that no detectable amount of erythropoietin disappears from the medium of a 24 hour culture⁽²⁶⁾.

D. Time Course.

The most important feature of the time course is that after a lag of about 2 hours, labeled product stimulated by erythropoietin increases linearly with time^(17,19). There are 2 alternative explanations for this.

1. All sensitive cells start to respond to the hormone at the same time, and they incorporate glucosamine at a constant rate as long as erythropoietin is present. That the presence of the erythropoietin is required for the continued response was demonstrated in an experiment in which cells were removed from a medium containing erythropoietin

after 1 hour's incubation and transferred to medium without it, which had been suitably preincubated with other cells. The stimulated incorporation lasted from about 3¹/₂ to 7 hours of incubation. After that, these cells did not incorporate more glucosamine than controls not given erythropoietin from the start⁽²⁰⁾.

2. A second explanation might be that there is a constant rate of formation of sensitive cells from a pool of marrow cells that are potentially sensitive to erythropoietin. The response during the entire incubation period may be thought of as the sum of a number of short bursts of incorporation by the induced fraction of the marrow cell population. I am inclined to favor this explanation because it seems very unlikely that the same group of cells would continue to respond for 20 hours with a fixed rate of glucosamine incorporation. The results of the experiment* described in Fig. 2 support the hypothesis that a constant number of cells is responding to the hormone per unit time. The accumulated response to 3 different doses of hormone was measured at an early and a late time point in the incubation. Granting that the hormone level remains essentially constant in the culture medium, and observing that the quasi linear response range remains unchanged with time, within the limits checked, it is possible to conclude that the ratio between responding cells and hormone molecules remains the same during the whole incubation period. A constant stimulated rate was established for each hormone dose. The plots of accumulated stimulated product extrapolate for all doses to the same time point for 0 effect. If the number of responding cells had varied significantly during the incubation this relationship would not have held. For instance, had the number of responding cells decreased with time then the response to the highest dose (0.5u) would have fallen below its present value at 23 hours on both plots. This would have caused a break in the semilogarithmic plot and the time point for extrapolated 0 effect would have deviated from 4 hours.

If one follows the effect of erythropoietin added at 0 time to a culture past 24 hours of incubation one notices that the incorporation of label from ¹⁴C-glucosamine into the whole cell and its acid insoluble fraction no longer parallel each other⁽²⁷⁾. The stimulated incorporation into the whole cells continues, although no longer at a constant rate, to the end of the second day and ceases then. The rate of incorporation of ¹⁴C-glucosamine into the acid insoluble fraction of the cells is actually depressed in the erythropoietin samples from the second to the fourth day (Table 2). Perhaps a response to erythropoietin consisting of an increased influx of hexosamine and accumulation of its low molecular weight derivatives continues to be possible

as long as there are sensitive cells in the stem cell pool. On the other hand another part of the response could depend on the availability of macromolecular acceptors for the labeled carbohydrates. These may cease to be made by the cell after 1 day's incubation perhaps due to exhaustion of the medium. It should be pointed out here that there is already a precedent for an erythropoietin mediated accumulation of a precursor in cells. Hrinda and Goldwasser⁽²⁸⁾ have reported recently on the increased Fe uptake of bone marrow cells caused by erythropoietin at a time before increased heme synthesis becomes measurable. This process is dependent on new protein synthesis.

As has just been stated, bone marrow cells cease to respond to erythropoietin added at 0 time at the end of the second day of culture. This is not because potentially sensitive cells cannot survive past this time. Even after 4 days of incubation cells do still respond to the addition of erythropoietin by incorporating additional glucosamine*. This response is if anything greater on the fourth day than on the first, although total cell number and control incorporation decrease drastically with time (Fig. 3).

My interpretation of these findings is that the erythropoietin sensitive cells are a small fraction of the total marrow cells, which are very resistant to the unfavorable conditions that they experience during 5 days of culture. They may be in a cell cycle of about 48 hours duration, and once triggered off into erythroid precursors cannot be replaced. After that length of time in the presence of erythropoietin they would have all been used up. Alternatively, they could have a shorter cell cycle, but after 48 hours incubation no more replenishment of the cell pool is possible.

I must emphasize here that the responses seen after 48 hours are once again only in terms of total cell uptake of label. Acid insoluble compounds could not be formed in increased amounts.

E. Inhibitor Studies.

I would like to turn now to the effect of inhibitors on the system. The effect of puromycin on 24 hour glucosamine incorporation into whole cells was to eliminate 95% of the incorporation despite the presence of the hormone⁽²⁰⁾. This suggests that protein synthesis is necessary for the expression of erythropoietin action in terms of glucosamine incorporation. This is also true for stimulated ⁵⁹Fe incorporation into hemoglobin⁽²⁹⁾ and ⁵⁹Fe uptake per se⁽²⁸⁾ in marrow cells.

Krantz, Gallien-Lartigue and Goldwasser^(30, 31) have shown that actinomycin D inhibits the erythropoietin effect on RNA and hemoglobin synthesis. This inhibitor also prevents the stimulated uptake of glucosamine by the cells⁽²⁰⁾. The response to actinomycin added at the same time as erythropoietin was dependent on the ratio of amount of inhibitor to the number of cells. 0.25 μg per million cells completely abolished the effect of erythropoietin. However, 0.156 μg actinomycin per million cells, (1.25 μg actinomycin- 8×10^6 cells/ml) caused no change at all in incorporation in the first 4 hours of incubation. In the next 3 hours, the stimulated incorporation was reduced by about 40% in the presence of actinomycin and soon thereafter ceased altogether. I should add here that it is known from Krantz's⁽³⁰⁾ work that 0.16 μg actinomycin per 10^6 cells added at the beginning of the culture did not abolish all RNA synthesis immediately, but by 6 hours it was down to 10% of its original value.

A study of the effect of various actinomycin concentrations on the 24 hour glucosamine incorporation due to erythropoietin revealed dose response curves exhibiting an initial region of high sensitivity to low drug concentrations followed by a region of reduced further response to increasing drug concentrations⁽³⁾.

It is possible to ascribe the 2 slopes observed in these curves to 2 or more processes based on DNA dependent RNA synthesis ultimately affecting glucosamine incorporation. Reich⁽³²⁾ has observed a similar 2 slope curve in a study of the actinomycin inhibition of purified RNA polymerase in the presence of DNA primer. One can formulate a hypothesis that the smaller amount of inhibitor stops production of inducible cells by preventing the sensitization of stem cells to erythropoietin in a process requiring RNA synthesis. The stimulation of already existing sensitized cells would continue until they were exhausted. This would lead to the intermediate degrees of inhibition already described. A large amount of actinomycin would prevent not only sensitization but also the processes involved in the response to the hormone.

In experiments in which the actinomycin was added at the fifteenth hour of incubation, that is, at a time when the erythropoietin effect was fully established, the erythropoietin stimulated glucosamine incorporation continued unaffected for 3.5 hours and then stopped abruptly⁽²⁰⁾. If one assumes that in this case the inhibitor had an immediate effect on m-RNA synthesis, then the 3 to 4 hour persistence of stimulated glucosamine incorporation suggests a similar maximum life time of m-RNAs related to that. The proteins coded

R

C

R

C

A

for are probably enzymes transferring activated carbohydrate moieties, or protein acceptors for the carbohydrates, or both.

The effect of the mitotic inhibitor colchicine was tested in order to see whether cell division plays any part in erythropoietin induced glucosamine uptake⁽²⁰⁾. Colchicine at the 10^{-7} M level had no effect whatsoever on the 23 hour uptake of glucosamine. This must be contrasted with findings by Gallien-Lartigue⁽³¹⁾ that the same amount of colchicine under the same conditions greatly reduced erythropoietin stimulated hemoglobin synthesis. These data can be reconciled by referring to the hypothesis that stimulated glucosamine incorporation involves only a relatively short burst during a part of the cell cycle⁽²⁰⁾. It would not be affected by the arrest of mitoses as long as the generation of cells in the proper phase of the cycle continued. Hemoglobin synthesis, on the other hand, goes on more or less continuously and is normally amplified by cell division. Mitotic arrest there prevents much of the stimulated hemoglobin synthesis.

Experiments were performed utilizing the incorporation of thymidine into the acid insoluble constituents of cells⁽²⁷⁾. It was found that addition of erythropoietin to cultures caused an increased rate of DNA synthesis to appear within 3-4 hours. The overall DNA synthesis rate fell off rapidly in control cultures; the presence of erythropoietin retarded this decrease in synthetic rates. The erythropoietin stimulated increment disappeared after about 20 hours of incubation. It reappeared later in long term cultures, seemingly during periods when the control DNA synthesis was at a minimum. The increased rate of DNA synthesis due to erythropoietin could be the consequence of preparation for division by those cells that had been triggered off for differentiation by the hormone. This would be so if (as is likely) their cycle was shorter than that of undifferentiated stem cells. Alternatively, or in addition, the replenishment of the stem cells themselves could lead to increased DNA synthesis in the culture. It may be questioned whether there is a need for active DNA synthesis in a culture to permit the initiation of erythropoietin action. Therefore the effect of erythropoietin was followed by measuring both glucosamine and thymidine incorporation.

Hydroxyurea, an inhibitor preventing the conversion of ribonucleotides to deoxyribonucleotides, was employed to reduce DNA synthesis⁽²⁷⁾.

It was established that 10^{-3} M hydroxyurea reduced DNA synthesis to 2% of its normal value in a 2 hour period at the beginning of the incubation. At the same time, glucosamine incorporation was essentially unaffected.

Another sample, 24 hours later, from the same group of cultures showed DNA synthesis in hydroxyurea containing samples to be 12% that of controls. The shift from 2 to 12% was due to the pronounced fall off of DNA synthesis in the controls. The erythropoietin had been added at 0 time. Whereas the rate of DNA synthesis with and without hydroxyurea was very different, the stimulated increase of glucosamine incorporation into the acid insoluble fraction was similar in terms of counts incorporated, in both sets. From this and other experiments it must also be noted that hydroxyurea did not prevent the erythropoietin stimulated increase of DNA synthesis either.

It seems then that a very low rate of DNA synthesis, such as was caused by hydroxyurea, does not preclude erythropoietin action or even seriously diminish it. This observation unfortunately does not decide whether or not DNA synthesis is needed at all. Obviously some cells are continuing to synthesize DNA, even when unable to make deoxy-ribonucleotides, probably by scavenging pathways utilizing preformed deoxyribonucleosides. It is impossible at this time to say whether or not these are the cells that respond to erythropoietin by increased glucosamine incorporation, but it is perhaps significant that at least some of these cells do respond with increased DNA synthesis.

Other Hormones.

In addition to erythropoietin, the sex hormones seem to be involved in the direct regulation of erythropoiesis. Near physiological amounts of estradiol administered to male rats caused a reduction of red cell formation suggesting a direct effect of the steroid on the hemopoietic tissues⁽³³⁾. More recently Jepson and Lowenstein⁽³⁴⁾, from studies of ⁵⁹Fe incorporation into erythrocytes of polycythemic mice, suggested that the utilization of erythropoietin by the marrow cells was blocked by estrogen. Much higher doses of estrogen, employed by Mirand and Gordon⁽³⁵⁾, interfered also with the production of erythropoietin in the test animals.

Androgens cause an increase in erythropoiesis, but the mechanism involved is still unclear. Naets and Wittek⁽³⁶⁾, in studies of the ⁵⁹Fe incorporation of polycythemic mice, found that androgens either potentiate the action of erythropoietin by increasing the number of stem cells, or their sensitivity to erythropoietin, or else they influence a step in erythropoiesis after stem cell differentiation. Fried and Gurney⁽³⁷⁾ and Mirand and Gordon⁽³⁵⁾ suggest additionally that erythropoietin production itself is also increased by androgens.

Summary.

Certain features of erythropoietin action⁽³⁾ are recapitulated in Fig. 4. The first part of the figure shows the stem cell cycle. Cells may, after completing a full turn of the cycle, divide into 2 new stem cells, or they may, under the influence of erythropoietin, leave the cycle. It has been shown⁽²⁰⁾ that the response of an unsynchronized population seems to be made up of incremental responses distributed over a long time. This suggests that it is only during a certain phase of the cycle that stem cells can interact with erythropoietin. From experiments with actinomycin inhibition, one may postulate, although this is far from certain, a process of sensitization to erythropoietin involving DNA dependent RNA synthesis. This would be characterized by a greater actinomycin sensitivity than the rest of the cellular RNA synthesis.

The actual induction process follows the entry of the hormone into the cell. Erythropoietin seems to act as a derepressor in the sense of Jacob and Monod⁽³⁸⁾. It seems to cause the transcription of genetic information, hitherto unexpressed, from nuclear DNA into m-RNA⁽³⁹⁾ (Krantz⁽³⁰⁾ observed the synthesis of new m-RNA like material 15 minutes after adding erythropoietin to a culture). The newly initiated process of cellular differentiation or specialization includes the synthesis of RNAs related to hemoglobin, iron transport, glucosamine incorporation into various cellular materials, and probably many others. It cannot take place in the presence of actinomycin. Obviously new protein synthesis is also essential for this specialization since when it is inhibited by puromycin or actidion no increased synthesis of hemoglobin or cell constituents labeled by ¹⁴C-glucosamine can take place.

Cell divisions are naturally involved before the final mature red cell can appear. But experiments with colchicine showed that the synthesis of additional stromal material is completed, and hemoglobin synthesis well on its way before the necessity for cell division arises.

Table I

Summary of Purification of Erythropoietin⁽⁹⁾

Fraction	Units/mg Protein	% Recovery	Purification Factor
Anemic Plasma	0.007	100	-
DEAE cell., XE-97 fraction (step III)	2.7	35	388
Ammonium sulfate fraction	19	20	2,700
Sulfoethyl Sephadex fraction	133	10.4	19,000
Calcium phosphate gel eluate	6500	2.2	930,000

Table 2

Response to Erythropoietin by Cells in Culture

Incorporation of
¹⁴C-glucosamine into

Incubation Time

	0-8 hours	16-24 hours	44-52 hours	70-71 hours	94-95 hours
1. Whole cells	+	+	+	0	0
2. Acid insoluble	+	+	-	-	-

In every instance erythropoietin was added at the start of the incubation.

+ = increase over control incorporation rate.

- = decrease from control incorporation rate.

0 = no change due to erythropoietin.

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Footnotes

On p. 5 *P. P. Dukes unpublished results.

On p. 7 *P. P. Dukes and E. Goldwasser unpublished results.

On p. 8 *P. P. Dukes and E. Goldwasser unpublished results.

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Legends for Figures

- Fig. 1. Relationship of amount of erythropoietin (step III) and stimulated ^{14}C -glucosamine uptake into rat bone marrow cells in culture ($18 \cdot 10^6$ nucleated cells/ml).
- Fig. 2. Relationship of amount of erythropoietin (step III) and stimulated ^{14}C -glucosamine uptake into rat bone marrow cells at 6 and 23 hours of culture (dose response on the left, time response on the right).
- Fig. 3. Increased incorporation of ^{14}C -glucosamine into rat bone marrow cells due to 0.25 u/ml erythropoietin added after various intervals of preincubation.
- Fig. 4. Scheme of erythropoietin action. SC= stem cells. Subscripts refer to stages in the cell cycle. E= cells in the erythroid line. The broken lines terminating in X show steps inhibited by the compounds indicated. GA= cellular material labeled by glucosamine. Hb= hemoglobin.

Figure 1

Figure 2

Figure 3

Figure 4