CONGENITAL ERYTHROPOIETIC PORPHYRIA is a rare metabolic disease. Its central feature is the excessive production of porphyrins by the erythroid cells of the bone marrow. The porphyrins deposit in the skin where they interact with light, heat and trauma to produce recurrent tissue destruction and scarring. Since available therapy is inadequate to prevent progressive scarring, the possibility was investigated that suppression of erythropoiesis by induced polycythemia might decrease porphyrin production.
and related tissue damage. As a part of this investigation, the relationship of erythrokinetics to porphyrin metabolism was studied during periods of active and suppressed marrow function.

**MATERIALS AND METHODS**

The patient, a 13-year-old girl, had mutilation of her fingers and disfigurement of her face from multiple prior porphyric lesions. Advice to avoid sun exposure, the use of protective clothing, several sun-screening preparations and trials of purines had all failed to prevent the appearance of new bullae. The patient had not been splenectomized. A full description of her clinical course, as well as some metabolic and hematologic studies, were previously reported.2

**Experimental Outline**

Following a baseline period of five days, the patient's hematocrit was increased by whole blood transfusion to 50 per cent and maintained at this level for 16 days during which a total of 670 ml of red cells were administered. Thereafter, her hematocrit was decreased by phlebotomy and plasma replacement to 25 per cent; this level was maintained for 19 days by removal of a total of 530 ml of red cells. Finally, a hematocrit of 60 per cent, achieved by packed red cell transfusion and removal of equivalent plasma volume, was maintained for 16 days during which a total of 1510 ml of red cells were administered.

Five ml of blood were drawn at 8 a.m. daily for microscopic examination, hematocrit determination and porphyrin assay. Urine, collected continuously, was frozen at the end of each 6-hour interval. All stools were collected and frozen. A bone marrow aspirate was obtained at least three times during each period of altered hematocrit.

**Routine Hematologic Measurements**

Microhematocrits were determined in duplicate. Reticulocyte percentages were made from blood films stained with brilliant cresyl blue and counted to 5000 red cells. These percentages were corrected for red cell concentration (corrected per cent reticulocytes = uncorrected per cent reticulocytes × hematocrit/39). The percentages of polychromatophilic red cells were each obtained from counts of 100 fields on a Wright's stained blood film; these percentages were also adjusted for red cell concentration. One smear from each marrow specimen was stained for iron by the method of Bothwell and Finch3 and for carbohydrate by the periodic acid Schiff (PAS) method of Pierce.4

**Iron Kinetic Procedures**

Ten μCi of 59Fe with a specific activity of about 10 μCi/μg were incubated with 10-15 ml of normal plasma and then injected intravenously as described elsewhere; a portion was saved as reference. Blood samples were obtained at intervals for 3 hours. Plasma radioactivity was measured on 2-ml samples in a well-type scintillation detector and related to the activity injected. The 100 per cent plasma radioactivity was determined by plotting counts versus time on semilog paper and extrapolating to zero time. The calculations of plasma and erythrocyte iron turnovers utilized the mean hematocrits of all samples and the plasma iron concentration at zero time with corrections for mean body hematocrit and trapped plasma.5 Erythron iron turnover was obtained by subtracting nonerythron turnover (a function of plasma iron and plasma volume)6 from the plasma iron turnover. Frequent radioactivity measurements with a collimated in vivo probe were made over sacrum, liver and spleen.

**Pigment Analyses**

Red cell porphyrins were determined by a method that incorporated minor modifications into a commonly used procedure.7 Porphyrins were extracted by adding whole blood to 10 volumes of acetone containing 2 per cent by volume of concentrated hydrochloric acid.
Table 1.—Erythropoiesis and Porphyrin Excretion Data

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<th>Period</th>
<th>Normal</th>
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<tr>
<td></td>
<td>Baseline Times</td>
<td>Polycythemia Times</td>
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<tr>
<td></td>
<td>Normal</td>
<td>Baseline</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>60</td>
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<tr>
<td>Reticulocytes (%)</td>
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<td>1.4 †</td>
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<td>Erythroid:myeloid ratio</td>
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<tr>
<td>Plasma iron turnover</td>
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<tr>
<td>Erythron iron turnover</td>
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<td>1.9</td>
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<tr>
<td>Total porphyrin excretion (mg./24 hours)</td>
<td>75</td>
<td>35 †‖</td>
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<td>Total porphyrin excretion/mg. of plasma iron turnover †‖</td>
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<td>2.4</td>
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<tr>
<td>Total stereobilin excretion (Approximate mg./24 hours)</td>
<td>50</td>
<td>110</td>
</tr>
<tr>
<td>Total stereobilin excretion/mg. of plasma iron turnover †‖</td>
<td>3.5</td>
<td>1.6</td>
</tr>
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</table>

* Data are from the second period of polycythemia with exception of stereobilin data which is from first period.
† Doubly corrected for the longer average reticulocyte life span indicated by the presence of polychromasia.
‡ Calculated from the assumption that baseline plasma iron turnover was the same as in a 1962 study, and utilization was 29 per cent at 6 days. Baseline blood volume assumed to be 66 ml./Kg.¹²
§ Additional data: average serum iron 114 µg./100 ml.; plasma volume 37 ml./Kg. indicating a whole blood volume of 77 ml./Kg. in this period.
‖ Additional data: average serum iron 63 µg./100 ml.; plasma volume 51 ml./Kg. indicating a whole blood volume of 65 ml./Kg. in this period.
* Calculated from the data of Hosain et al.⁹, for the patient's weight (39 Kg.) assuming a blood volume of 66 ml./Kg.¹²
** Erythron iron turnover corrected for changes in blood volume.
†† Data from the same day as plasma iron turnover.
†‖ These calculations use the total body iron turnover/24 hours, namely baseline 31 mg., polycythemia 14.5 mg., and anemia 67.7 mg. A comparable normal would be about 18 mg./24 hours.

The mixture was stirred thoroughly, filtered and the residue washed with acetone-HCl. An equal volume of ethyl acetate was added to the combined filtrate and the uroporphyrin extracted with 10 per cent sodium acetate. In subsequent extractions, coproporphyrin was removed with 0.1 N HCl and protoporphyrin with 3 N HCl. Uroporphyrin and coproporphyrin were purified by adjusting each solution to pH 3–4, transferring the porphyrin to ethyl acetate, washing with water and finally re-extracting with 1.5 N hydrochloric acid for fluorometric measurements.

Urinary porphyrins were analyzed by the method of Schwartz et al.⁷ Porphyrin analyses of the thawed stools were performed by the method of Rimington et al.⁸ The average hourly fecal porphyrin outputs were calculated and then distributed in midnight to midnight periods.

Sterecobilin was determined as stereobilinogen by the method of Schwartz et al.⁹ using the extinction coefficient of Henry et al.¹⁰ These excretion values were also distributed in midnight to midnight periods.
Fig. 1.—Erythropoietic parameters. PIT indicates the times at which the two plasma iron turnover studies were done. Per cent nucleated erythroid cells are calculated from total marrow nucleated cells while per cent fluorescent normoblasts and marrow fluorescent reticulocytes (the non-nucleated marrow cells with cytoplasmic fluorescence) are expressed as a per cent of the total marrow nucleated erythroid cells.

Fluorescent Microscopic Observations of Blood and Bone Marrow

Unstained blood smears were examined under the fluorescent microscope with a No. 53 barrier filter for erythroid cells containing porphyrins. The source of illumination was a Zeiss 200-watt mercury lamp with a BG 12 filter. All of the examinations were made some months after the smears were prepared but at a time when the fluorescence level was quite stable and mainly represented uroporphyrin.*

*On fresh blood and marrow smears, approximately half of the total fluorescence faded rapidly. Protoporphyrin is known to be quite labile, particularly in the presence of ultraviolet light; coproporphyrin and uroporphyrin are more stable. The extremes of blood coproporphyrin were 0.08–0.25 µg./ml. of whole blood, only a minor part of total blood porphyrin.
Urinary erythropoietin was measured by injecting dialyzed urinary concentrates into

\[ \text{Urinary erythropoietin} \]

Fig. 2.—Tissue iron radioactivity patterns. The abscissa indicates both the days after iron injection (1 through 7) and the days of hospitalization (34 through 40 and 49 through 56).

The fluorescent cells in peripheral blood smears exhibited the typical orange-red color of porphyrins; however, nonfluorescent cells were difficult to distinguish in the dark blue background. To circumvent this problem, the average number of red cells was first determined under white light in several representative oil emersion fields. Then, under ultraviolet light, the fluorescent cells in 50 similar fields were counted and the percentage adjusted for red cell concentration.

Unstained specimens of aspirated marrow were first scanned under ultraviolet light with a 40-power objective. Ten areas were circumscribed on each of two slides; these areas were chosen close to the particles but where cells were sufficiently separate for identification. After the nucleated and non-nucleated fluorescent cells in each area were counted, Wright's stain was applied, and the total number of nucleated erythroid and nonerythroid cells was determined in the same area. In addition, the appearance of numerous specific cells was compared under both viewing conditions.

Erythropoietin

Urinary erythropoietin was measured by injecting dialyzed urinary concentrates into
Fig. 3.—Diagrammatic representations of the correlations between erythroid maturation and degree of porphyrin fluorescence. Fluorescence intensity is indicated by the density of stippling. Abbreviations: O.N., orthochromatic normoblasts. P.E., polychromatophilic erythrocytes. E., erythrocytes.

polycythemic protein-starved mice. Erythropoietic activity in the assay animal was determined by injecting radioiron and comparing the amount incorporated into red cells with that of a standard of known activity. By this method, normal subjects average 4.0 units per day with a range of 1.2–9.5 units.

RESULTS

Erythropoiesis

Measurements relating to the patient’s erythrokinetics are shown in Table 1 and in Figs. 1 and 2. Her baseline rate was somewhat above normal (Table 1). Plasma iron turnover (PIT) was 31 mg./day (normal 18 mg./day), the erythroid:myeloid ratio was 1:1, and the absolute reticulocyte count was 2.8 per cent. Changes with induced polycythemia and anemia are shown in Figs. 1 and 2. Erythropoietin disappeared within 12 hours after the induction of polycythemia (day 40). Corrected reticulocyte values decreased to approximately 0.2 per cent. Both the PIT and the per cent of marrow nucleated erythroid cells decreased to about half of the original level. Studies of the in vivo distribution of radioiron within the body (Fig. 2) showed an accumulation within the marrow, i.e., the sacrum. With induction of anemia, erythropoietin became elevated and erythropoiesis increased. The corrected reticulocyte count reached a peak of 8 per cent and the plasma iron turnover was 67.7 mg. per day, twice the basal level and about four times normal. There was an increase in marrow normoblasts to 40 per cent (erythroid:myeloid ratio of 3:1). Ferrokinetic studies showed normal internal iron kinetics, except for some accumulation of radioiron in bone marrow and spleen.

Fluorescence of Erythroid Cells

Porphyrin fluorescence was found only in the late normoblast and young reticulocyte stages of erythroid development (Fig. 3). In each marrow preparation the percentage of fluorescent normoblasts was highly correlated with the number of cells in the later normoblast stages. The intensity of fluorescence varied with the maturation stage. The youngest fluorescing cells had a faint, barely perceptible pink color limited to the nucleus. During cell maturation, fluorescence increased and became apparent in the cytoplasm. The orthochromatic normoblasts, especially their nuclei, were a bright orange-red. The non-nucleated marrow cells showing fluorescence were found to be polychromatophilic on Wright's staining, the degree of polychromatophilia being parallel to the intensity of cytoplasmic fluorescence. In the peripheral blood.
Fig. 4.—Pigment parameters. Calculations are described in the text. The distribution of porphyrins between urine and stool was normal with essentially all the uroporphyrin appearing in the urine, and about 90 per cent of the coproporphyrin plus all of the protoporphyrin appearing in the stool; 10–16 per cent of the urinary porphyrin was coproporphyrin. Approximate total normal daily porphyrin excretion for a child of the same weight (39 Kg.): 1.2 mg., (uroporphyrin 0.01 mg.; coproporphyrin 0.29 mg.; protoporphyrin 0.90 mg.). Total urinary porphyrins 0.1 mg./24 hours (0.004 mg./hour); total stool porphyrins 1.1 mg./24 hours.

the per cent of polychromatophilic erythrocytes followed that of the fluorescent reticulocytes shown in Fig. 1, but reached a peak of only 0.4 per cent. In some instances, fluorescence was associated with the debris within the reticuloendothelial cells of the marrow. Such phagocytic cells, readily located during the control period, were virtually absent during polycythemia and were seen only in the last marrow specimen of the anemic period. Nuclear vacuoles and cytoplasmic granules, previously described in congenital erythropoietic porphyria, were seen in all marrow preparations of this study but were limited to the more mature normoblasts. Stainable iron was also present in all of the marrow aspirates. There was no PAS staining material in the erythroid
cells* and no poikilocytosis (an indicator of ineffective erythropoiesis) in the peripheral blood smear.

The cell maturation stage at which fluorescence was first seen appeared to be the same in each marrow sample. Therefore, the changes found in the per cent of fluorescent normoblasts reflected changes in the relative number of normoblasts in the later maturation stages. In the basal state, 16 per cent of the total marrow normoblasts fluoresced. With the induction of polycythemia, the percentage increased to 90 per cent. Accompanying this increase in relatively more mature forms, there was an increase not only in the per cent of normoblasts with nuclear vacuolation but also in the number of vacuoles per nucleus. Fluorescent reticulocytes disappeared from the peripheral blood but were found in increased numbers within the marrow. With the induction of anemia, normoblast fluorescence and nuclear vacuolation returned to the basal state. Fluorescent reticulocytes decreased in the marrow and increased in the circulating blood. Occasional nucleated red cells, absent from peripheral blood films during the other periods, were seen on all except the first day of the anemic period.

Quantitative Measurements of Porphyrins and Stercobilin

Pigment measurements are summarized in Table 1 and Fig. 4. Throughout the study parallel changes in uroporphyrin and coproporphyrin occurred in blood, urine and stool, respectively, with changes in the stool values delayed about 24 hours. Following the induction of polycythemia, the high levels of uroporphyrin and coproporphyrin decreased rapidly to about one-half the baseline. With the induction of anemia, uroporphyrin and coproporphyrin began to rise on the fourth day to a mean level about twice basal. Protoporphyrin had a similar but slower pattern of response. Blood protoporphyrin declined during polycythemia and rose gradually with anemia. The basal porphyrin excretion (75 mg./day) was about 60 times normal (1.2 mg.). At all levels of erythropoiesis there appeared to be a constant relationship between plasma iron turnover and total porphyrin excretion.

Stercobilin excretion apparently followed a different pattern. Like porphyrin excretion, it approximately doubled during the anemic period; however, it did not significantly decrease with polycythemia.

DISCUSSION

Since Schmid, Schwartz and Watson15 first noted that porphyrin fluorescence was largely confined to erythroid cells in congenital erythropoietic porphyria, the erythron has been considered the site of the abnormal porphyrin production. Furthermore, several authors16-20 have indicated that most of the erythroblasts fluoresce. In studies of this feature in five cases, Schmid et al.13 showed that fluorescence was found primarily in the late erythroblasts, that “pronormoblasts or erythroblasts showed little fluorescence, basophilic normoblasts somewhat more,” and that 30–70 per cent of the total normoblasts fluoresced.

*Larizza14 reported PAS material in erythroid cells from one case of congenital erythropoietic porphyria.
However, they also reported that “some normoblasts in all developmental stages failed to exhibit any red fluorescence.” They noted that both the fluorescing and nonfluorescing erythroblasts tended to occur in separate clumps together with their own kind. These findings were interpreted as evidence for two cell populations in this disease. Indeed, the data presented in Fig. 2 on per cent of normoblasts which fluoresce can be interpreted as indicating a “normal” population is suppressed during polycythemia, whereas a “porphyric” population is not suppressed.21 However, more extensive examinations of the marrow smears for fluorescent erythroblasts and, after staining, for erythroblast and late erythroblast numbers in the same circumscribed areas, indicate that fluorescence is limited to late normoblasts and that all cells at this stage of development appear to fluoresce. We have, therefore, concluded that in this patient there is only a single population of erythroid cells, all of which produce excess porphyrins.

A previous episode of transient erythroid hypoplasia in this patient had been associated with a marked decrease in urinary porphyrin excretion.2 The possible relationship between erythropoiesis and porphyrin output led us to induce anemia and polycythemia in the hope that porphyrin output might be modified through normal regulatory mechanisms and that this might have therapeutic implications.

During the control period, the patient had a basal level of erythropoiesis of one and one-half to twice normal (Table 1). The change in erythropoiesis from the basal level was an approximate 50 per cent decrease during polycythemia and an approximate 100 per cent increase during anemia. At all levels of erythropoiesis (baseline, polycythemic, and anemic) the PIT, EIT and corrected reticulocyte count were in reasonable accord with each other, especially if the reticulocyte poolshifts mentioned below are taken into account. However, the erythroid:myeloid ratio is consistently greater than twice the other parameters. The explanation for this discrepancy is not apparent, but the erythroid:myeloid ratio is an indirect calculation dependent on a normal white cell mass.

During polycythemia, the in vivo counting showed a marked accumulation of radioactive iron in the erythroid marrow which was probably augmented by the decreased needs of erythropoiesis and the excess iron introduced into the erythron R-E circuit by transfusion. The much milder accumulation of radioiron during anemia was probably minimized by the negative iron balance created by phlebotomy; removal of iron from the erythron R-E circuit would increase the recirculating of red cell iron through the R-E cell.

In these studies there was clear evidence of change in erythropoiesis induced by altering the hematocrit of the circulating blood. The suppression induced by transfusion, however, was not marked. Birkhill et al.22 appeared to be more successful in normal human subjects transfused to hematocrit values of 50–56 per cent; at 2–3 weeks, only 3–5 per cent of normoblasts were seen in marrow aspirates. Our results are more similar to reports of patients with thalassemia or hemoglobinopathy transfused to near normal levels.23–27 Chaplin et al.28 in the only report of prolonged maintenance, found that in three patients with
sickle cell anemia, reticulocyte counts averaged less than 0.4 per cent. However, in the only patient with marrow examinations after 3 months of transfusion-maintained polycythemia, the marrow showed a normal or only slightly decreased erythroid population. In another report, elevation of the hematocrit to 60 per cent in an attempt to control the crisis in a patient with SC disease failed to reduce the red cell precursors by more than 60–70 per cent.29 In other situations where hypoxia has been corrected, erythropoiesis has been only partially suppressed.30,31 Overall, these studies suggest that in man it may be difficult to suppress the marrow completely by transfusion-induced polycythemia.

Of interest are the quantitative aspects of porphyrin production in comparison with the varying levels of erythropoiesis. Throughout the study there was a constant relationship between porphyrin production and the utilization of the iron by the marrow. Calculations show that for every mg. of iron leaving the plasma, approximately 2½ mg. of porphyrin were excreted (Table 1). Since this ratio was constant at all levels of erythropoiesis, it is likely that changes in the amount of porphyrin excretion reflected changes in erythropoietic activity. Approximately 65 per cent of the total porphyrin production at baseline was required for hemoglobin synthesis and 35 per cent was excreted as excess.* Changes in erythropoiesis led to a somewhat more rapid response in urinary porphyrin excretion than in reticulocyte count. This finding is consistent with previous estimates indicating that a large portion of the excreted porphyrins are derived from the erythroid cells of the marrow15 rather than from the fluorescent reticulocytes in circulation,f and further that uroporphyrin and coproporphyrin are rapidly released from the erythroblasts soon after their synthesis.

The effects of alteration in the erythropoietin level on red cell maturation and the early destructive process in this patient are of particular interest. It is known that erythropoietin not only increases the number of cells undergoing maturation but also accelerates the process. This effect is due to a shortening of the mitotic interval,32 an increase in rate of hemoglobin synthesis,33 and an

*23.4 ml. red cells/Kg. x 39 Kg. = 910 ml. R.B.C. mass; 910/120 = 7.6 ml. R.B.C./day; 7.6 x 0.3 Gm. hemoglobin/ml. = 2.28 Gm. hemoglobin/day; 2.28 x 3.5 per cent = 0.08 Gm. protoporphyrin/day for a normal child of the same weight. Thus, porphyrin formation for hemoglobin synthesis needs approximately 140 mg. at baseline. Considering porphyrin excretion of 75 mg./day, 140 mg. = approximately 65 per cent of the total porphyrin production (140 + 75 = 215 mg./day).

†Although in this study the red cells and plasma were not analyzed separately, many previous analyses have shown that about 75 per cent of the circulating protoporphyrin and about 25 per cent of the uroporphyrin are in the patient's red cells. Based upon this distribution at baseline her red cells had about 8 μg. of porphyrin/ml. If this is viewed as being entirely in the fluorocyte, 0.4 per cent fluorocytes indicate a porphyrin concentration of 2 mg./ml. fluorocytes. Since this is a mean value, the entering fluorocytes would probably have a value about twice this, or 4 mg./ml. As previously calculated, a normal child of the same size would be expected to produce 7.6 ml. R.B.C./day, and since the patient's baseline is about 1½ times normal, she would be producing about 12 ml. R.B.C./day; 12 x 4 = 48 mg. porphyrin. Thus, calculations of the maximal erythrocyte porphyrin entering the peripheral blood daily cannot account for the total daily porphyrin excretion.
CONGENITAL ERYTHROPOIETIC PORPHYRIA

early release of erythroid cells from the marrow. The observation that porphyrin fluorescence occurred only in the late normoblasts and young reticulocytes provided a unique way of examining alterations in maturation in this patient. With induction of anemia, the shift of fluorescent reticulocytes from the marrow to blood was consistent with the early release of reticulocytes associated with erythropoietin stimulus. During polycythemia, the confinement of fluorescent reticulocytes to the marrow represents an opposite process. The associated increase in late (fluorescent) normoblasts relative to other erythroid forms suggests a delay of enucleation due to decreased erythropoietin.

Several isotopic studies have shown that, in this condition, the majority of labeled stercobilin appears within the first 2 weeks after giving labeled glycine rather than the normal appearance of 80 per cent of the stercobilin at the end of the red cell life span. The rather small discrepancy between PIT and EIT suggests that this so-called "early-labeled" stercobilin is due to the turnover of heme within the erythron. While the nearly identical changes of several measures of erythropoiesis at different levels of production indicate that no qualitative changes had occurred in the relationships between proliferation and hemoglobin synthesis, the tissue radioactivity patterns and the stercobilin excretion suggest that there was a difference in the handling of a portion of the heme. During polycythemia, there was a relatively greater retention of iron in the marrow and relatively greater excretion of stercobilin. There was also a relatively greater quantity of erythroblast nuclear vacuoles which have previously been shown to contain heme. Perhaps the delay in nuclear extrusion during polycythemia allows for the continued excessive production of heme which accumulates in the vacuoles and is eventually catabolized.

ACKNOWLEDGMENTS

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REFERENCES


*Note is taken of the apparently opposite change observed by Runge and Watson in bovine congenital erythropoietic porphyria. After bleeding, the proportion of marrow erythroblasts which fluoresced increased.

*Although some stercobilin undoubtedly arose from senescent transfused cells, this was unlikely a major source of stercobilin, especially during the first period of polycythemia which lasted only 16 days and without indication of hemolysis.
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Congenital Erythropoietic Porphyria. II. The Effects of Induced Polycythemia

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