Destruction of Immature Erythrocytes Measured by Bilirubin Excretion

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In accelerated erythropoiesis, many immature reticulocytes appear in the peripheral blood, but it is uncertain whether they all mature to normocytes.

Evans' suggested from mathematical analysis of this problem that short-lived erythrocytes exist even in normal subjects. The formation of short-lived erythrocytes during erythropoietic stimulation was also suggested by Berlin² and Neuberger,³ while London et al.⁴,⁵ analyzed hemoglobin formation and bile pigment metabolism using radioactive glycine and found evidence for the presence of short lived erythrocytes in normal subjects.

Stohlman⁶,⁷ studied survival of red cells by a labeling method and also by measuring the size distribution of red cells in severe anemia caused by acute bleeding or phenylhydrazine. He also concluded that some erythrocytes were short-lived. We studied the production of these short-lived red cells biochemically⁸ and karyometrically.⁹

However, it is difficult to demonstrate unequivocally the early destruction of newly formed erythrocytes by any of the technics used previously, because samples are contaminated with bilirubin derived from other sources besides erythrocyte hemoglobin and because not all the labeled red cells are in the peripheral circulation.

Therefore, to confirm the existence of short-lived erythrocytes, studies were made on early labeled bilirubin in normal and anemic rats using glycine 2-¹⁴C and ¹⁴C-labeled erythrocytes.

Materials and Methods

A. Experimental Animals

Sprague Dawley and Wistar strain male adult rats, weighing about 220 Gm. were used. Animals were fed on a standard diet ad libitum. The red blood cells, hemoglobin, reticulocytes and white blood cells of these animals were within the normal ranges before experiments.

B. Experimental Methods

1) Reticulocyte formation. Two ml./100 Gm. body weight of blood was drawn by cardiac puncture under ether anesthesia to induce reticulocytosis.

2) Blood transfusion. Donor rats injected with glycine 2-¹⁴C were anesthetized with...
ether. Fifty U. of heparin was injected and as much blood as possible was withdrawn from them. One ml. of this whole blood was transfused into recipient rats via the femoral vein. To avoid hemolysis, the erythrocytes were not washed with saline solution.

3) Insertion of bile fistula. Following Waldeck's method, a polyethylene tube was inserted into the common bile duct of rats under ether anesthesia. Subsequently these animals were given food and water ad libitum and vitamin K, (0.02 mg.) was injected subcutaneously every other day. About 10 days after the operation, when the bile flow became constant, glycine 2-14C or 14C-labeled erythrocytes were injected intravenously.

C. Assay Methods

1) Determination of erythrocyte 59Fe utilization. To obtain the accurate ferrokinetic data the blood sample should be as small as possible. Following Huff's method, a dose of 10 μ Ci of 59FeCl3 in saline solution was administered intravenously, by which no radiation injury was not detected in the period of observation. To measure the rate of iron utilization the radioactivity in 0.01 ml. samples of blood was measured at intervals in a well type scintillation counter.

2) Determination of bilirubin in bile. Twenty four hour bile samples were collected in the dark in test tubes immersed in ice. A small amount of liquid paraffin was placed in the tube to avoid contact of the bile with air. Bilirubin was measured by a modification of Mallory and Evelyn's method using the bilirubindiazo reaction.

3) Extraction of bilirubin from bile. Bilirubin was extracted by the method of Ostrow et al. The specific activity (S.A.) of labeled bilirubin obtained by this method was the same as that of bilirubin obtained by Gray and Whidborne's method in which bilirubin was recrystallized after alumina column chromatography.

RESULTS

1) Changes in Bile Pigment Excretion Following Bleeding (Fig. 1)

The amount of bile pigment excreted per day into the bile drainage tube became constant within 10 days after the operation. When these animals then suffered acute hemorrhage due to cardiac puncture, the rate of bilirubin
excretion decreased temporarily to about two thirds of the normal value and then returned to the normal range within 10 days.

2) Rate of Utilization of Iron in Red Cells and Bilirubin Production after Bleeding

The total radioactivity (total c.p.m.) and S.A. of bilirubin and the rate of utilization of $^{59}$Fe in red cells in rats with bile fistulae under control and anemic conditions were determined over a 10 day period after injection of glycine 2-$^{14}$C ($10 \mu$ Ci/100 Gm. body wt.) and $^{59}$FeCl$_3$ ($10 \mu$ Ci/animal).

a) Utilization of iron in red cells (Fig. 2). In normal animals, utilization of iron in red cells showed a relatively slow increase up to 75 per cent utilization without detectable oscillation, but in anemic animals, utilization increased rapidly to 80 per cent or more within one day after hemorrhage and then fluctuated greatly for a few days.

b) Bilirubin production after hemorrhage (Figs. 3, 4 and 5). The total c.p.m. and S.A. of bilirubin were always higher in animals after hemorrhage than in control animals. Moreover, after hemorrhage (Nos. 4 and 6) there was a second slight rise in c.p.m. and S.A. which was clearly separated from the first peak.

3) Bilirubin Formation due to Red Cell Destruction (Figs. 6 and 7)

The destruction of newly formed erythrocytes in the peripheral blood was studied as follows. Glycine 2-$^{14}$C ($200 \mu$ Ci/animal) was injected intraperitoneally. Blood was collected after 10 days from control rats and after 4
days from anemic rats. One ml. of these highly labeled blood specimens was given to rats with bile fistulæ, assuming that the former contained labeled normocytes and the latter contained labeled reticulocytes. The total c.p.m. and the S.A. of bilirubin originating from labeled reticulocytes were higher than those derived from labeled normocytes, and values were highest 6 days after injection of blood. Results on bilirubin formation in Wistar and Sprague Dawley rats are shown in Figures 6 and 7. The rate of red cell destruction was estimated by comparing the total radioactivity in heme in erythrocytes with the total radioactivity in bilirubin.

With Wistar rats, the destruction rates of newly formed erythrocytes during maximum reticulocytosis and in the normal state were calculated as 14.1 per cent and 12.7 per cent and as 1.8 per cent and 2.5 per cent in 7 days respectively. With Sprague Dawley rats, the values of the former were 3.2 per cent and 5.2 per cent and those of the latter were 0.87, 1.08 and 1.30 per cent in 7 days. As no radioactivity was detected in the bilirubin on transfusion of labeled plasma, all the radioactivity in the bilirubin seemed to originate from labeled erythrocytes.

**DISCUSSION**

It is generally thought that the reticulocyte is intermediate between the erythroblast and mature erythrocyte (normocyte) and maturates into a normo-

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**Fig. 3**—Labeled bilirubin production in normal rats injected 10μCi/100 Gm. body weight of glycine 2-14C.
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Fig. 4.—Labeled bilirubin production in anemic rats injected 10μCi/100 Gm. body weight of glycine 2-14C 24 hours after bleeding.

cyte through synthetic action. Erythropoiesis has mainly been studied morphologically, autoradiographically and ferrokinetically. Lajtha15 reported that proerythroblasts originating from stem cells grow into orthochromatic erythroblasts through four divisions, and that there is another series of cells in which some basophilic erythroblasts grow to maturity without cell division. Alpen5 introduced the concept of terminal division in the regulatory system of erythrocyte kinetics.

In contrast, Stohlman17 suggested the possibility of ineffective erythropoiesis in which erythroblasts are destroyed during their differentiation.

When there is an increased demand for erythrocytes, abnormal maturation of erythroblasts may increase but effective erythropoiesis seems to be more reasonable than ineffective erythropoiesis.

From biochemical studies on bone marrow,6 in reactive erythroid hyperplasia, proerythroblasts and basophilic erythroblasts with nuclei which are very active metabolically accumulate in the bone marrow. Quantitative karyometric studies on erythroblasts indicate that the reticulocytes appearing in the reticulocyte-crisis may originate from intermediate erythroblasts, and that the large-sized reticulocytes, which were thought to be formed by abortion of more immature erythroblasts, may be fragile and short-lived. But there is no direct evidence for these hypotheses. It is thought that almost all the protoporphyrin moiety of hemoglobin is converted to bilirubin and so is not reutilized.4,5,19

The mean time for conversion of injected hemoglobin into bilirubin is re-
ported to be approximately 3 hours, so this conversion is very rapid. Furthermore, it is reported that conversion of a small dose of \(^{14}\)C-hemoglobin, not exceeding the binding capacity of plasma haptoglobin, to \(^{14}\)C-bilirubin is nearly complete.\(^{19}\) Therefore, to determine the survival time and rate of destruction of peripheral red cells appearing in response to erythropoietic stimuli, the amount of bilirubin which was rapidly formed from hemoglobin and almost completely excreted without reutilization was studied.

Excretion of bilirubin were studied following acute hemorrhage in rats with bile fistulae. After acute hemorrhage, the rate of bilirubin excretion decreased to about two thirds of the normal value, and then returned to the normal level within 10 days.

If all red cells had a fixed life span, then, judging by the increase in formation of young cells after the hemorrhage, the decrease in bilirubin excretion should be greater and last longer. The rapid restoration of normal bilirubin production after acute hemorrhage indicates that there is increased degradation of hemoprotein, irrespective of the age of the red cells.

This bilirubin may be derived from the following sources:

1) directly from hemoprotein other than hemoglobin in the liver.
2) from ineffective erythropoiesis.
3) from circulating erythrocytes.

Labeled bilirubin in excreted bile was measured following administration of glycine \(2-{^{14}}\)C to rats with a bile fistula. The total radioactivity and S.A. of the bilirubin were found to be higher in animals after hemorrhage than
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in control animals. Moreover, in these animals there were two peaks of excretion of labeled bilirubin. Suggestions have been made on the origin of the first of these peaks by London4 and others.5 Some authors20,21 found two fractions in this peak. The first was attributed to shunt bilirubin not related to hematopoiesis, while the second was thought to be related to hematopoiesis and to be mainly due to hemolysis in the bone marrow.

The overall generation time22,23 of erythroblasts has been estimated to be not more than five days. Our study on anemic animals showed that excretion of labeled bilirubin increased more than five days after administration of glycine 2-14C. Therefore, some of the newly formed erythrocytes may be destroyed after reaching the peripheral circulation. It is interesting to compare the changes in the radioactivity of peripheral erythrocytes and labeled bilirubin. To measure the changes in radioactivity of peripheral blood with time a large volume of 14C-labeled peripheral blood is needed. Therefore, the decay curve of labeled erythrocytes was estimated by measuring radioactive iron.

When animals were given radioactive iron two or three days after bleeding, the rate of red cell 59Fe utilization increased rapidly to about 80 per cent or more after two days, and then fluctuated for several days, suggesting the existence of short-lived cells. But, the decrease during this fluctuation did not correspond to the second peak of 14C-bilirubin excretion.

Furthermore, as Robinson21 stated that the quantitative relationship between early and late bile pigment formation was similar in physiologic and in accelerated erythropoiesis, the increase in early bile pigment does not necessarily

Fig. 6.—Labeled bilirubin excretion after the injection of newly formed erythrocytes labeled with glycine 2-14C during maximum reticulocytosis and in normal state (in Wistar rats). Nos. 12 and 13 represent the destruction of young red cells in reticulocyte rich blood. Nos. 10 and 11 represent the destruction of normal young red cells.
mean the increase of the destruction of newly formed erythrocytes.

To detect short-lived cells, the destruction of newly formed erythrocytes labeled in vivo with glycine 2-14C in anemic and normal rats was studied by the excretion of labeled bilirubin after transfusion of their blood into rats with a bile fistula. Labeled bilirubin excretion increased to a maximum 5 to 6 days after transfusion of blood from anemic animals but did not increase on transfusion of blood from normal animals. These findings confirm the possibility that some of the newly formed erythrocytes produced in acute hemorrhage are easily destroyed after reaching the peripheral circulation. In other words, the production of short-lived cells, "stress reticulocytes," contribute to poiesis was increased following hemorrhage.

The proportion of the red cells appearing after bleeding which were rapidly destroyed was calculated by comparing the radioactivity of total excreted bilirubin with the total radioactivity of heme transfused as whole blood. In Sprague Dawley rats, the destruction rate of these short-lived cells were estimated as about 4.4 per cent at the peak of reticulocytosis and as about 1.1 per cent in control animals in 7 days. In Wistar rats these values were both higher, being 13.4 per cent and 2.2 per cent, respectively.

It is unknown why this difference between Sprague Dawley and Wistar strain rats is so big, but it may be due to a difference in erythrocyte fragility and in response to hemorrhage. However, in both strains more than four or five times as many of the red cells formed in the peak of reticulocytosis were destroyed as normal red cells.

Fig. 7.—Labeled bilirubin excretion after the injection of newly formed erythrocytes labeled with glycine 2-14C during maximum reticulocytosis and in normal state (in Sprague Dawley rats). Nos. 44 and 45 represent the destruction of young red cells in reticulocyte rich blood. Nos. 41, 42 and 43 represent the destruction of normal young red cells.
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SUMMARY

The change in radioactivity of bilirubin with time was measured after injection of glycine 2-14C into rats with a bile fistula. The total radioactivity and specific activity of bilirubin were abnormally high in rats of which erythropoiesis was increased following hemorrhage.

When the newly formed erythrocytes labeled with glycine 2-14C in the peak of reticulocytosis were transfused into rats with a bile fistula, the excretion of labeled bilirubin increased rapidly.

The amount of newly formed erythrocytes destroyed within 7 days after transfusion into normal animals was calculated as 4.4 per cent of the total erythrocytes formed during maximum reticulocytosis and 1.1 per cent of those formed in the normal state in Sprague Dawley rats, while in the Wistar strain, the values were 13.4 per cent and 2.2 per cent, respectively.

These results provide direct evidence for increased production of short-lived erythrocytes during enhanced erythropoiesis, and the hemolysis of newly formed erythrocytes soon after they reach the general circulation may contribute to the production of shunt bilirubin.

SUMMARIO IN INTERLINGUA

Le alterationes occurrente in le curso del tempore in le radioactivitate de bilirubina esseva mesurate post le injection de glycina-2-14C in rattos con fistulas biliari. Le radioactivitate total e le activitate specific de bilirubina esseva anormalmente alte in rattos in que le erythropoiese esseva intensificate post hemorrhagia.

Quando le novemente formate erythrocytos marcate con glycina-2-14C al culmine del reticulocytose esseva transfusionate ad in rattos con fistula biliari, le excretion de bilirubina marcate accresceva rapidemente.

Le quantitate de novemente formate erythrocytos destruite intra 7 dies post le transfusion ad in animales normal esseva calculate como 4,4 pro cento del erythrocytos total formate durante le reticulocytose maxime e 1,1 pro cento de illos formate in le ratto normal in rattos Sprague-Dawley, durante que in rattos Wistar, le correspondente valores esseva 13,4 e 2,2 pro cento, respectivemente.

Iste resultatos provide directe evidentia in supporto del these de un augmentate production de erythrocytos a breve superviventia durante periodos de augmentate erythropoiese, e il es possibile que le hemolose de novemente formate erythrocytos texto post que illos arriva in le circulation general contribue al production de bilirubina de shunting.

REFERENCES

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