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Research methods in reticulocyte physiology

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TABLE OF CONTENTS

RESEARCH METHODS IN RETICULOCYTE PHYSIOLOGY

The purpose of the following paper is to describe some of the methods which have been used in studying reticulocytes and the results which have been obtained using these methods. In addition, a new method will be described which on the basis of preliminary experiments seems to be promising.

Apparently the cells which are now known as reticulocytes were discovered by Ehrlich. A description of this discovery is given by Smith (1): "Ehrlich, in the Charite Annalen for 1885 very briefly mentions the important fact that in anemic blood certain red corpuscles become stained with hematoxylin and with methylene blue. He was also able to demonstrate granules within the blood corpuscles with the last named dye." However, Ehrlich believed that this basophilic staining was due to coagulation necrosis of the stroma of the cell.

Smith (1) was probably the first to recognize the true significance of diffuse basophilic staining of red cells. His method consisted of microscopic examination of fixed dry smears stained with alkaline methylene blue. He used blood from cattle infected

with Texas cattle fever, a disease producing marked hemolysis of red cells. He noticed that as the anemia in these cattle progressed, the following progressive changes occured in the blood: (1) Enlarged corpuscles were noted. (2) I arge corpuscles were noted in which granules could be detected by the nuclear stains. (3) Some of the large corpuscles stained diffusely. (4) Nucleated red corpuscles or hematoblasts appeared in the peripheral blood. He attributed these changes to the release of immature cells into the circulation. He says that Gabritschewsky (2) using a similar method in anemic men found diffuse staining of corpuscles but not granules, and suggested that they may be corpuscles which had been arrested in a certain stage of development. Smith, however, s ill claimed credit for first recognizing the significance of these cells for the following reasons: "My own experience with human blood is limited to a single case, but is worth mentioning here. In January 1890 . . . . I as enabled to examine the blood of a case of purpura. In it I found a small number of diffusely stained corpuscles and I at once diagnosed them as newly formed corpuscles in accordance with my experience with Texas fever blood. The history of this case was presented to the Association of American Physicians by Dr. Prentiss in May 1890 with my diagnosis

of these staine d corpuscles. This therefore ante-dates Gabritschewsky's paper."

It was later shown by several authors that if the cells were expose(to certain stains while still wet, the substance cau ing basophilic staining precipitated out in the form of clumps or strands and took on the color of the stain used. See Downey (3) for references. With the discovery that the basophilic substance formed a reticulum with upravital stains, the cells became known as reticulocytes.

Without giving historical details, it has been shown that in the course of development of the erythro-cyte in the bone arrow, the normoblast in some manner forms non-nucleated red cells. When these cells are first formed, they contain large amounts of basophilic substance which forms a dense reticulum with supravital stains. As these cells become older, they gradually lose this reticulum and become mature, non-reticulated erythrocytes. Reticulocytes can therefore be classified as to stage of maturation on the basis of the amount of reticulum present. Riddle (4) uses the following classification: ++++ Very dense reticular substance practically filling the reticulocyte; +++ dense reticular

substance in form of band, wreath, or plaque; + + scattered clumps of reticular substance present; + scattered strands of reticular substance. Baar and Lloyd (7) use the method of Heilmeyer and Trachtenberg (reference not given). These authors apparently use five groups:

0 nucleated RBC; I ball shaped reticulum; II netwc k forms; III incomplete network; IV granular forms.

At some point in the above maturation sequence, the erythrocyte is released from the marrow into the peripheral blood stream. The exact stage at which this occurs has been the subject of much debate. In the normal individual, at least some cells are released as reticulocytes because these forms are found normally in the peripheral blood. Wintrobe (5) states that the evidence at the present time seems to indicate that most red corpuscles are fully matured when they leave the bone marrow. Thi view is also held by Heath and Daland (6). One of the reasons given by Wintrobe is that in a case of adequately treated pernicious anemia, the initial rise in reticulocyte count is replaced by a rise in RBC count with a drop in reticulocytes.

Baar and Lloyd (7) say that Minot and his collaborators (1928, exact reference not given) believe

that all the erythrocyte formed by patients with pernicious anemia during the initial rapid response to liver therapy are released as reticulocytes only if the initial red count is less that 2.5 million. However, they believe that if the count is greater than 2.5 million, some of the erythrocytes are released in the mature, non-reticulated stage. The reason for these conslusions is as follows: The formula

$$E_p\, r = \text{Retics per } mm.^3 \text{ at peak} - \frac{E_o\, r}{1 - r}$$

(where E_p is the red count at the peak of the response, E_o is the red count before therapy, and r is the decimal fracti n which expresses the percent of reticulocytes at the peak of the response) shoul be true if : (1) at the peak of the response all the cells which have been formed were delivered as reticulocytes, (2) none of these reticulocytes havematured, and (3) there has been no destruction of red cells during this period. They have observed that this formula holds only in cases of low initial red count. They conclude from this that the reason this formula does not apply in cases with a higher initial count is hat some of the cells are released as non-reticulated erythro ytes. Baar and Lloyd (7) disagree with this interpretation. They point out that in cases of pernicious ane ia, blood destruction actually

takes place rapidly. They believe that the formula holds true in cases with low initial counts because the destruction of erythrocytes occurs at the same rate as the release of reticulocytes. In cases of higher initial count, the rate of destruction is not so great, and even though all new cells are released as reticulo-cytes the formula still does not hold.

Baar and Lloyd (7) also state that if it could be demonstrated that all red cells in the bone marrow were reticulocytes, this would be strong evidence that all cells are released in this form. They say that Istomanowa (1926 exact reference not given) has succeeded in showing that all erythrocytes in the bone marrow of the rabbit are reticulated. However, they point out that all attempts to prove this for man have failed.

These same authors reason that if all cells are released as reticulocytes, an absence of reticulocytes should mean that no erythrocytes are being produced. They studied pigment excretion in several patients without circulating reticulocytes and found that the amount of hemoglobin actually destroyed correlated well with the amount calculated to have been destroyed on the basis of the amount of pigment excreted. They say that since these pigments are not re-used, their results suggest

that in the absence of reticulocytes, there is no re-generation of red cells. They then reason from this that all cells are released as reticulocytes. Young and Lawrence (8) sing Baar and Lloyd's method of study were unable to come to these conclusions.

The studies of Riddle (4) are interpreted by Baar and Lloyd as showing that "if erythrocytes are not all liberated from the bone marrow as reticulocytes there is at least a fixed mathematical relation between the daily intake of RBC and the number of reticulocytes." Details of these tudies are given below.

Riddle (4) noted that in patients with pernicious anemia who had recieved adecuate doses of liver extract the red count rose rapidly for a few days and then gradually leveled off. If the increase in red cells is plotted agains t days of therepy, the curve for non-reticulated cells is similar to that for total red cells, but the rapid rise occurs about 2 days later. He interpreted this as due to the maturation of the immature reticulocytes, a process which therefore must take two days. This figure for the life span of reticulocytes is also supported by the following interesting line of reasoning:

Many growth phenomena follow the general biological law of growth and regeneration whereby the initial rate of growth is slow, but increases gradually and then gradually decreases in a fashion similar to the manner in which it increased. Thus, if the amount of growth is plotted against time, a sigmoid curve is obtained (Fig. 1)

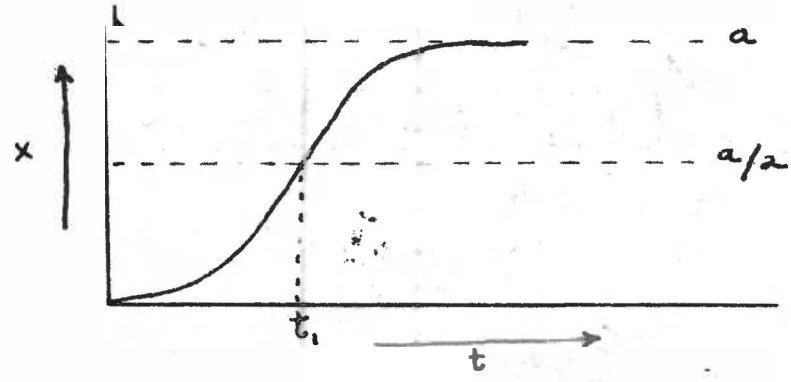

Fig. 1.

This can be represented by the equation

$$\log \frac{x}{a - x} = ka(t - t_1)$$

where x = amount of growth, t = time since growth began, a = limit of growth which x approaches, t_1 = the time at which x = a/2, and k is a constant determined for each application of the formula. Robertson (9).

The actual values of erythrocyte increase during the initial rapid response of pernicious anemia to liver therapy agree very closely with the growth curves calculated by the above formula. As an example, one

case gave the following formula:

$$\log \overline{1.02} \overset{x}{-} x = 0.54\ (t - 4.3)$$

where x is the increase in erythrocytes expressed as millions of red blood cells. It was then assumed that the increase in the number of reticulocytes released from the marrow, if none were maturing, should follow a similar curve with the same value for t_1. By substituting known values of x and t in the formula and using 4.3 for the value of x, it was found by trial and error that the values a = 1.2 and k = 0.45 worked best. This gave the formula:

$$\log \frac{x}{1.2 - x} = 0.54\ (t - 4.3)$$

They point out that the difference between the maximum increase in erythrocytes (a = 1.02) and the calculated maximum increase in reticulocytes (a = 1.2) may be due to the destruction of old erythrocytes during the response. Curve **A** (Fig. 2) was obtained when the above formula for the calculated number of reticulocytes produced by the marrow was plotted against the time after the therapy started.

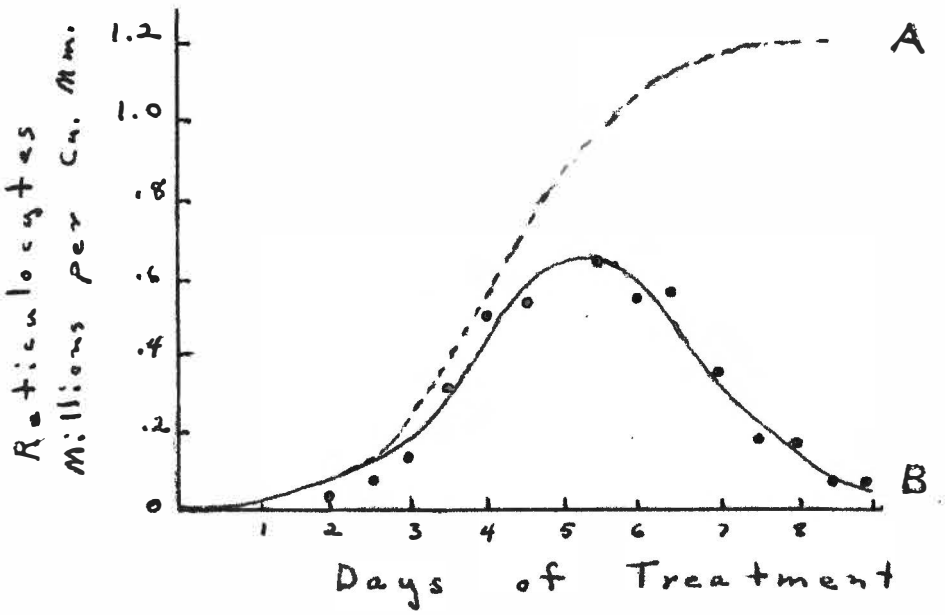

Fig. 2.

It was then reasoned that if the reticulocytes actually matured in 2 days, the actual reticulocyte concentration at any time should be equal to the value obtained from curve A at this time minus the value obtained from curve A two days previously. In other words, the reticulocyte concentration at any time should be equal to the number which the marrow has produced minus the number which has matured. It was then possible to plot a curve of the expected reticulocyte concentration at any time after therapy was started using only curve A. This is drawn as curve B in Fig. 2 and the actual observed reticulocyte concentrations are plotted in

the same figure. The author interprets the close correlation between the expected concentration and the actual concentration of reticulocytes as good evidence that the original estimation of a 2 day maturation time is correct. Four cases were used in this study. In two other patients with lower doses of liver extract, the time required for maturation was 3 to 4 days.

The in vitro maturation time of reticulocytes has been studies by Health and Dland (6). They incubated blood containing large numbers of reticulocytes at various temperatures and made reticulocyte counts on these incubated samples at regular intervals. As a source of blood from pernicious anemia patients at the peak of their response to therapy, and blood from rabgits made anemic by bleeding or intraperitoneal injection of phenylhydrazine. In addition to incubation in the test tube at 37 degrees, some samples of blood were incubated in the pleural cavities of rabbits. Maturation time in both cases was the same. These authors found that reticulocytes in vitro at 37 degrees and in the pleural cavity of the rabbit decreased at a regular rate over a period of from one to four days. They alsofound that the rate of disappearance for reticulocytes produced in human hemolytic anemia and

pernicious anemi.. and those produced in rabbits which were bled or tre.ted with phenylhydrazine was the same, and concluded from this that reticulocytes in both cases were of the sa-e general nature. These authors also observed granule whic'. stained both with brilliant cresyl blue and Wright's stain in some of the erythro-cytes and reticulocytes of blood which had been incubated or refrigerated. They speculate that these granules appear in vivo in the course of development of the cell.

Young and Lawrence (8) studied the maturation of human reticulocytes in vivo. Their method consisted of transfusing a patient with aplastic anemia with large quantities of blood containing a very high percentage of reticulocytes. The source of blood for transfusion was a patient wi.h an atypical hemolytic anemia. This patient consistantly had reticulocyte counts between 45 and 73 percent. The time required for maturation in the patient was compared with the in vitro maturation time. They found that these reticulocytes gradually matured over a period of about 140 hours, and that all the reticulocytes were destroyed in 8 days. They also found that the in vivo results correlated well with the in vitro maturation time. These authors point out the important fact that the reticulocytes which they studied

were not produced by a normal individual and refrain from assuming that the normal maturation time is the same.

Nizet (10) studied the time required for reticulocytes with different amounts of reticulum to mature. He also found close agreement of in vivo and in vitro results. A possible reason for the different values which various authors have reported for maturation time is suggested by the experiments of Plum and his co-workers. A summary of these studies is given by Plum (11). They have found that plasma and liver extracts contain certain substances which accelerate the ripening of reticulocytes in vitro. These substances are also found in other tissues, but highest concentrations are found in plasma, marrow, and liver. The concentration of these substances in a sample of plasma is expressed by these authors as the "ripening index". They have found that there is an inverse relationship between the ripening index and the reticulocyte count is humans, and that species which have high reticulocyte counts have low concentrations of reticulocyte ripening substances, and vice versa. They believe that "the

reticulocyte level is fixed by the ratio between the intensity of erythropoisis and the concentration of the ripening substance." They also point out that other unknown factors may influence the level.

The ease with which the reticulocyte level may be determined has made it a valuable clinical sign of hemopoisis. However, correlation of reticulocyte level and actual rate of red cell production has been impossible because of two reasons: (1) The exact time required for the maturation of reticulocytes is not known, and (2) the percentage of newly formed cells which are released as reticulocytes is unknown. It is likely that these values vary with the rate of red cell production, or at least with different clinical conditions. Accurate determination of in vivo maturation time and the percentage of cells released as reticulocytes in various clinical conditions and at various rates of erythropoisis would be not only a valuable a dition to our knowlege of reticulocyte physiology, but would also be clinically useful in calculating the rate of erythropoisis from the reticulocyte level. With these goals in mind, an attempt is being made to study the release and maturation of reticulocytes by using radioactive iron

as a tracer and autoradiography as the means of determining which cells contain the tracer substance. The following method should tell what percentage of red cells are released from the bone marrow as reticulocytes:

A small dose of Fe^{59} is administered intra-venously to the subject to be studied. At frequent intervals, samples of venous blood are withdrawn, mixed with anticoagulant, and immediately centrifuged. About 20 drops of the plasma is withdrawn from the tube and placed in another small test tube. The cells are then re-suspended in the remaining plasma and one drop of the red-cell mixture is placed in the tube with the 20 drops of plasma. This gives 21 drops of blood with a red cell concentration of approximately 1/20 that of the original sample. Twenty-one drops of reticulocyte stain made according to the method of Brecher (12) is mixed with the diluted blood and the mixture is allowed to stand for 10 minutes, allowing the reticulocytes to become stained. The mixture is then shaken, and a small drop is placed on a slide, spread, and allowed to dry. The dried film is then placed on the stage of a compound microscope having a marker in the objective, oil immersion lens, and a graduated mechanical stage. A small area of the slide is systematically examined in

autoradiogram, the coating of emulsion must be as thin as possible. Painting the slide with a liquid emulsion would be rapid, but the resulting coating is usually too thick and uneven. A product is available from the Eastman Kodak Company which greatly simplifies the operation, and gives a very uniform coating of emulsion only 5 microns thick. The product is known as Kodak Autoradiographic Stripping Emulsion, and consists of a strip of cellulose acetate film 35 mm. wide. This film is coated with a layer of clear gelatin 5 microns thick, and then with a 5 micron layer of special photographic emulsion. This emulsion contains high concentration of silver halide particles, and is designed specifically to detect beta radiation. The clear gelatin protects the layer of emulsion from scratches as it is stripped from the cellulose acetate film, and makes the emulsion easier to handle when it is removed from the film. To coat the slide with this emulsion, the following steps are performed in a darkroom using a Wratten series 2 safelite at a distance of at least 4 feet:

(1) the roll of stripping film is removed from its protective package and pieces the desired length are cut from the roll. 2) The emulsion and protective latin are removed from the cellulose acetate film by

grasping a corner of the emulsion with a pair of forceps and slowly peeling it off. If the emulsion has a tendency to curl up after removal from the film, breathing on it will cause it to flatten out.

(3) The gelatin-emulsion layer is then turned over and placed on the surface of a pan of distilled water, emulsion side down. It will float on the surface and will be wrinkled at first, but will become perfectly smooth after about 3 minutes. (4) The slide with its fixed film of blood is then placed in the pan of water, and moved until it is directly under the emulsion. A small camel-hair brush may be used to move the emulsion on the surface of the water so that it lies in the proper position over the slide.

The slide is then lifted straight up out of the water, removing the emulsion from the surface of the water. This causes the emulsion to lie perfectly flat over the blood film and to wrap around two sides of the slide since the piece of emulsion is nearly twice as wide as the slide. (5) The coated slide is then allowed to drain and is dried in a stream of air from an electric fan. The slide is leaned against an object such as a box or the wall during these stages to prevent damage to the wet emulsion. (6) The dried slides are then placed in a small light-

proof slide box of black plastic. A small test tube filled with anhyirous calcium chloride and stoppered loosely with cotton is placed in the slide box and the lid is sealed with black plastic electrician's tape.
(7) The sealed box of slides is then placed in a refrigerator to allow the radioactive cells to "expose" the emulsion. The exact time required for exposure varies widely and depends upon the amount of radioactivity present in the cells. The exposure time is probably best determined by trial and error.

Developing the exposed autoradiograms is carried out as follows. All solutions should be kept at 18 degrees centigrade:
(1) The slide is developed in Kodak D-19 for 4 minutes.
(2) It is then washed in distilled water for 10 seconds.
(3) Fixing is done by placing the slide in Kodak F-5 fixing-hardenin· solution for 10 minutes.
The slide is then air-dried and examined under the same microscope used to locate the reticulocytes. The location of the black areas in the emulsion which indicate the location of radioactive cells is then plotted on the same chart as the reticulocytes. It should be obvious from examination of the chart whether the newly released and therefore radioactive cells are present on the slide

as reticulocytes or as non-reticulated red cells, provided the smear was taken before the radioactive reticulocytes matured.

Similar techniques can probably be devised to study the maturation time of reticulocytes also. A method which would probably give good results would be to plot the location of both reticulocytes and radioactive cells and calculate the percentage of new (radioactive) cells which contain reticulum. This percentage could then be plotted against time on rectangular coordinates. (Fig. 4) The initial portion of the graph should be horizontal at a level determined by the percentage of cells which are released as reticulocytes. As soon as the new reticulocytes begin to mature (point B Fig. 4) the percentage will decrease. The time from point A when radio-active cells are first detected in the blood to point B when the percentage of radioactive cells containing reticulum first begins to decrease would be the shortest maturation time for this subject's reticulocytes.

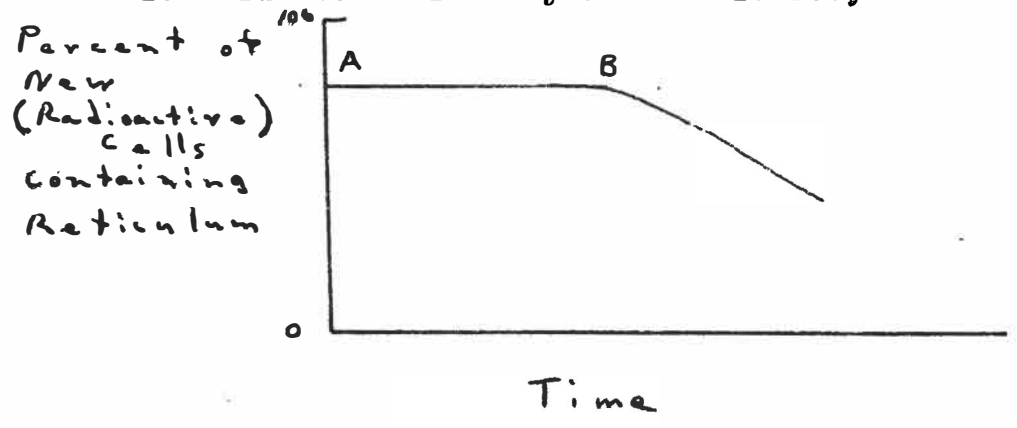

Fig. 4

The experiments described above could probably be refined by simply noting the amount of reticulum present in each cell as it was plotted on the chart. It should also by possible to use C^{14} labeled glycine instead of Fe^{59}.

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