

THE EFFECTS OF EXPERIMENTAL PLETHORA ON BLOOD PRODUCTION.

By OSWALD H. ROBERTSON, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 22, 1917.)

Our present knowledge concerning normal blood production indicates that the bone marrow functions at a constant rate supplying new red corpuscles to replace those lost daily in the process of normal blood destruction. Since an extra loss of blood causes greater activity of the bone marrow,¹ it has seemed possible that were the constantly occurring normal loss compensated for artificially there would be no necessity for the production of new cells, and consequently a lessened activity of the bone marrow might result. In an attempt to bring about this condition, rabbits have been made plethoric by repeated small transfusions of blood and the effect of the procedure on the bone marrow has been observed.

Some work has already been done in this direction. Hess,² with the purpose of determining the effect of plethora on the heart, gave rabbits repeated large transfusions. At the end of 2 months of plethora the rabbits were killed. In examining the bone marrow, he claimed to have found evidence of a markedly diminished activity. The erythroblasts and myelocytes were decreased in number, and there was a fibrous hyperplasia present. Itami³ gives a more detailed account of these findings, agreeing with Hess in his conclusions. Boycott and Douglas⁴ carried on a similar series of experiments but failed entirely to confirm Hess's and Itami's results, which seem to have been obtained in only a few animals.

¹ The term "bone marrow activity," as used in this paper, refers only to the erythropoietic function of the marrow.

² Hess, R., *Deutsch. Arch. klin. Med.*, 1909, xcv, 482.

³ Itami, S., *Folia hematol.*, 1908, vi, 425.

⁴ Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 414.

The criteria which these two sets of investigators used as evidence of depressed bone marrow activity, that is, marked histological changes in the marrow, may account to some extent for their contradictory results, since the red marrow of even normal rabbits varies much in its morphology as indicative of blood production and also in its distribution.

In the present work, use has been made of an indicator which may be thought to represent more nearly the actual functional variations in the marrow taken as a whole; namely, the number of reticulated red cells in the circulating blood. In addition, the number of these cells in the bone marrow itself has been determined. In normal animals, reticulated red corpuscles are present in the blood in fairly constant numbers. There is good evidence that increased activity of the bone marrow is accompanied by an increase in the percentage of these cells, and that the percentage roughly parallels the degree of hyperfunction.^{5,6} *A priori*, one would suppose that if the activity of the bone marrow were lessened, a drop in the reticulated cell count would result.

Method of Producing Plethora.

The method of producing the plethora has already been described⁷ but will be briefly given here. Rabbits were used. Each recipient was provided with three to six donors of the same hemolytic group. Transfusions of 10 cc. of blood were made daily. The donors were used in rotation, so that each one lost at most only 10 cc. every 3 days. In this way the production of any considerable anemia in the donor rabbits was avoided. In some of these rabbits, the blood loss was made up so promptly that the hemoglobin percentage did not vary throughout the period of the bleedings; in others it dropped slightly. The blood for transfusion was obtained from the donor by cardiac aspiration into a syringe containing 1 cc. of a 1 per cent solution of sodium citrate in normal salt solution. This small amount of citrate, when well mixed with 10 cc. of blood, was sufficient to prevent coagulation for the few minutes required to introduce the blood into the marginal ear vein of the recipient. The recipient rabbits for the most part weighed from 1,500 to 1,800 gm. All were young rabbits. Both sexes were employed.

⁵ Lee, R. I., Minot, G. R., and Vincent, B., *J. Am. Med. Assn.*, 1916, lxxvii, 719.

⁶ Vogel, K. M., and McCurdy, U. F., *Arch. Int. Med.*, 1913, xii, 707.

⁷ Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

The Sahli hemoglobinometer was used for determining the degree of plethora. The inaccuracies of this method are recognized, but dependable readings were obtained by diluting for the color comparison after the test mixture had stood exactly 5 minutes. In some of the animals, the red cells were counted and the color index was estimated.

Method of Counting Reticulated Cells.

The following method for counting the reticulated cells was found to be preferable to those previously described. A saturated solution of brilliant cresyl blue was made up in normal salt solution. This was kept as a stock solution. When a count was to be made, a small quantity of it was diluted 80 times⁸ with normal salt solution and mixed with blood in a pipette for counting white cells in the proportion of one part of blood to twenty parts of cresyl blue solution. The mixture was shaken in the pipette for 5 minutes. The cells were thus equally distributed as well as stained. They were counted at once in fresh preparations, which were sealed with vaseline to prevent disturbances due to drying. At least 1,000 red cells were counted at each test. When the number of reticulated cells was less than 1 in 1,000, 10,000 red cells were counted. In the latter case, only the first 1,000 were counted individually, the field being the unit of count for the remaining 9,000.

For several days before transfusions were begun, the number of reticulated cells was determined daily in those rabbits destined to be recipients.

In a large number of normal rabbits examined, the reticulated cells were found to vary for the most part between 10 and 20 per 1,000 erythrocytes. Rarely they were as many as 30 or fewer than 5 per 1,000. Only two animals showed less than 5 per 1,000, one having a count of 3 and the other of 4. The variation in the individual from day to day may be slight or, relatively speaking, considerable.

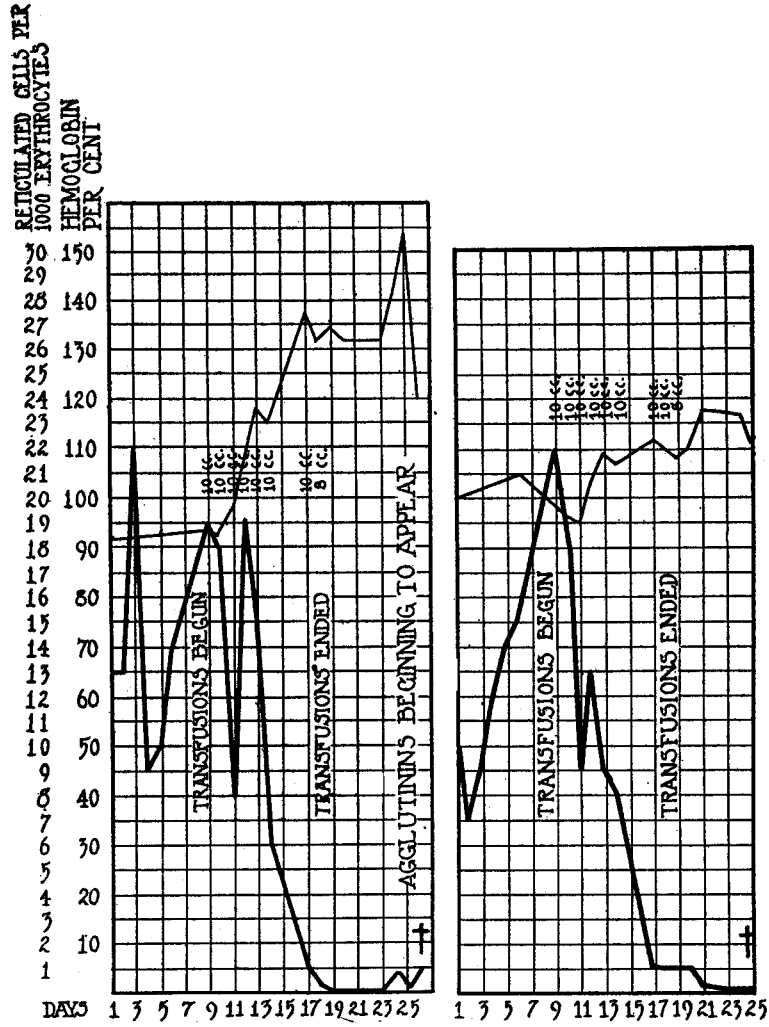
⁸ Since doing this work, a second saturated solution of cresyl blue has been made up, using a different stock of the dye, which went into solution to a considerably greater extent than the first. The result was that a 1:80 dilution of this saturated solution was much too strong a staining fluid. It was found necessary to dilute to 180 for satisfactory staining. It is apparent, therefore, that each saturated solution of cresyl blue has to be tested beforehand for its optimum staining dilution. This is a very simple matter and needs to be done only once.

Production of Plethora.

Sixteen rabbits were rendered plethoric by the method described. The rate at which the hemoglobin per cent increased in different animals varied considerably. In some it rose rapidly. In others it increased slowly. The degree of plethora ultimately attained likewise varied much. An amount of blood which in one animal resulted in a rise of fifty points in the hemoglobin per cent, produced in another individual of the same size a rise of only thirty points. For this reason some of the animals were transfused more often than others. Even so it was often found impossible to force up the hemoglobin per cent in these refractory individuals to a height easily reached in other animals. The shortest period of transfusion was 9 days, during which the animal was given seven transfusions; the longest, 20 days with nineteen transfusions. As a rule, the hemoglobin per cent rose from a normal of 80 to 90 per cent to 140 to 150 per cent, at which point it remained fairly constant despite the continued introduction of blood.

No notable change in the percentage of reticulated cells occurred until after several transfusions. Then, as plethora became well defined, the number of reticulated cells in the circulating blood began to diminish (Text-figs. 1 and 2). This decrease in number was sometimes rapid and soon became marked. Often the reticulated cells practically disappeared. Counts below 1 in 10,000 were frequent (Text-fig. 1), and sometimes a search through a whole slide would reveal none. The charts of Text-figs. 1 and 2 will illustrate the change that occurred in eight of the sixteen recipients. In five others, the drop in reticulated cells was less marked, though definite. They decreased to about 1 in 1,000. The degree of plethora was fully as great in these animals as in the afore mentioned ones, and no reason has been found for the differences observed.

Anomalous results were obtained in three rabbits. One showed only a slight drop in the reticulated red cells (Text-fig. 3). In the remaining two, these cells failed to decrease at all. One had a purulent pneumonia, with some anemia, despite the transfusions. The other developed an increased number of reticulated cells for which no cause was ever found.



TEXT-FIG. 1.

TEXT-FIG. 2.

— Reticulated cells.
 — Hemoglobin.

It is evident from these results that a diminution in the number of reticulated cells in the circulation follows almost constantly the production of an artificial plethora. A possible influence of sodium citrate in causing this change has been ruled out by observations in five rabbits which received daily injections of sodium citrate alone, in some cases twice as much being given as in the rabbits made plethoric. The number of reticulated cells did not decrease in these animals.

Examination of the Bone Marrow.

The bone marrow from five of the eight rabbits in which the reticulated cells dropped markedly was examined for its content of these cells. The following procedure was employed.

Red marrow from the upper and lower ends of the femur was taken into a mixture of equal parts of Locke's solution containing $\frac{1}{4}$ per cent of gelatin and an isotonic watery solution of sodium citrate. In this mixture clotting does not occur, and even the most delicate cells are well preserved. The gross appearance of the marrow was normal except for a congestion such as was present in all the other organs. A small portion of it was teased on a slide in a drop of the Locke's-citrate mixture and a count made at once from the preparation. Then, with a view to washing out the cells of the marrow, the needle of a syringe was thrust into it here and there, and gelatin-Locke's solution injected under considerable pressure. In this way a large part of the substance of the marrow was washed out. The washings were then centrifuged and counts made on the sedimented cells according to the technique used for the peripheral blood. To obtain a figure for purposes of comparison, an average was taken from the counts on the teased specimen and the washings.

The average number of reticulated cells in the bone marrow of the five plethoric animals was 14 per 1,000 red cells, the highest number 22 and the lowest 5 per 1,000. In striking contrast are the figures obtained in the examination of five normal rabbits. These showed an average of 320 per 1,000 red cells, the highest 540 and the lowest 160 per 1,000.

Anemia Following Plethora.

In certain of the plethoric rabbits there occurred, after many transfusions, a sudden marked drop in hemoglobin. In one instance (Text-fig. 4), despite daily transfusions of 10 cc. of blood, the hemo-

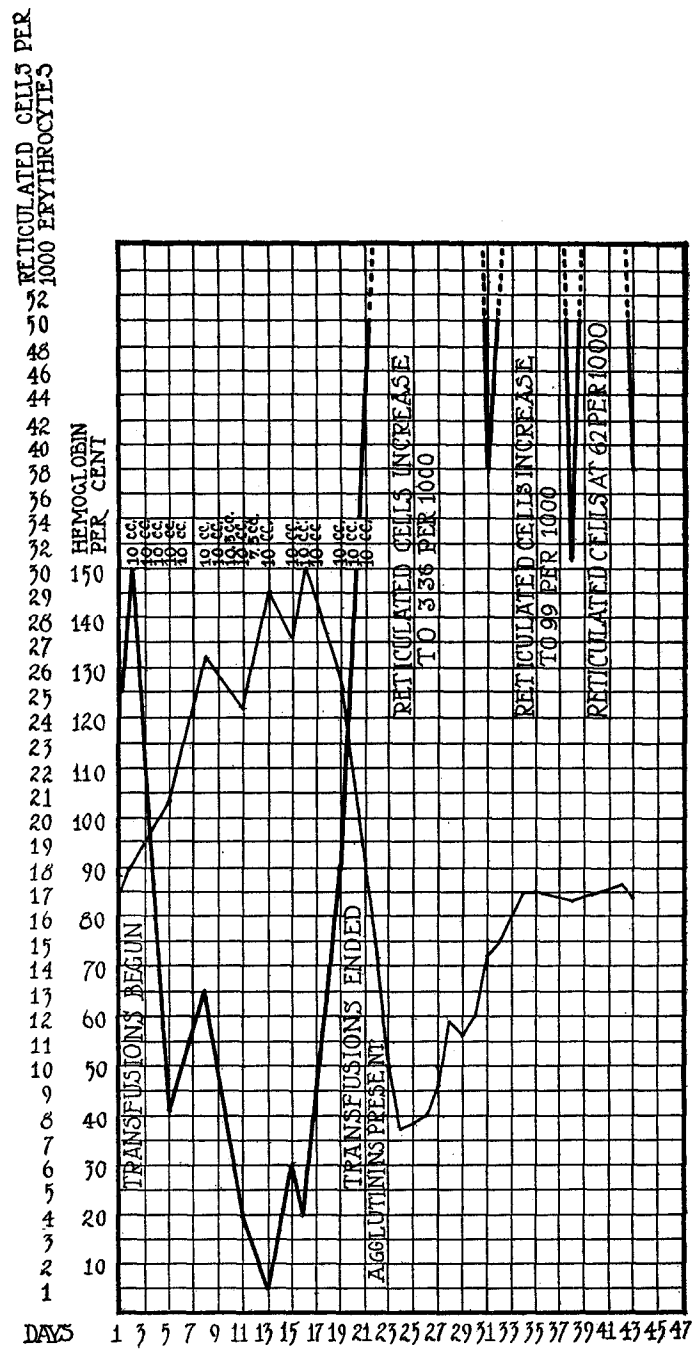
globin fell in 6 days from 150 per cent to 75 per cent. Transfusions were discontinued in such animals, and there rapidly ensued a severe anemia. This was observed in three rabbits. Charts of two of them are shown in Text-figs. 4 and 5. The cause of the rapid transition from plethora to anemia has not yet been determined, but it is significant that isoagglutinins for the donors' blood developed in all three rabbits at about the time when the hemoglobin first began to fall. The simplest explanation would seem to be that, following a number of transfusions, a lytic process develops against the strange cells, and they are rapidly destroyed. This results not merely in a return of the hemoglobin to normal but in an anemia, because of the peculiar condition of affairs indicated by the drop in reticulated red cells during the plethora. During it the bone marrow activity sinks far below normal, whereas blood destruction is maintained at the normal rate at least. Thus, little by little, as the animals' own cells are destroyed and not replaced, the bulk of the circulating blood comes to be strange blood liable to destruction when the lytic principle develops. The extent to which the blood cells proper to the recipient have been destroyed and replaced by alien cells is suddenly revealed through the destruction of this alien blood.

The anomalous behavior of one rabbit, whose chart is shown in Text-fig. 3, strongly supports this explanation of the phenomenon. In this animal there came at length a drop in the hemoglobin, but instead of progressing to an anemia, as in the three rabbits described above, it sank to a point only slightly below normal. The failure of anemia to develop here would seem to be due to the fact that during the plethora, blood production had been maintained at almost its normal rate as indicated by the lack of any but a slight diminution in the number of circulating reticulated cells.

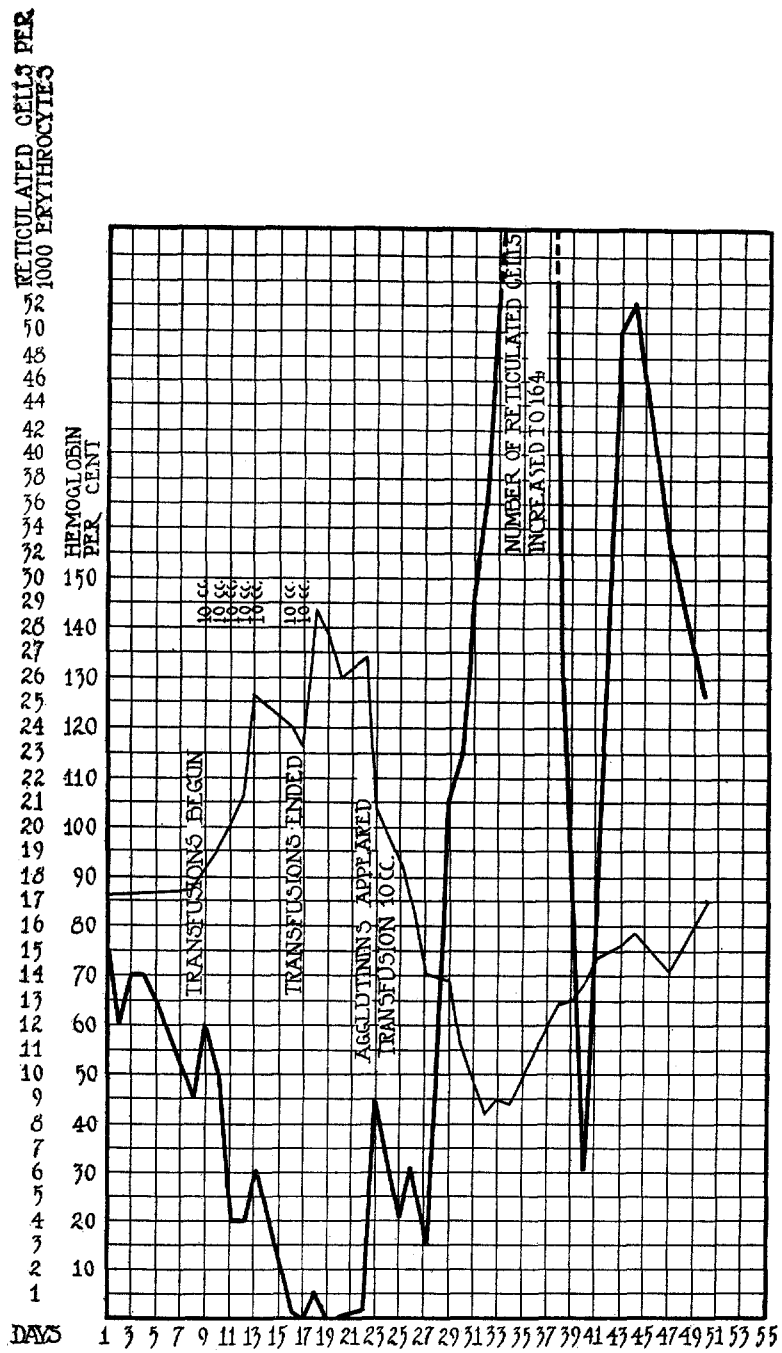
The recovery from the anemia in the three rabbits just mentioned was exceedingly rapid. A similar rapid recovery was noted by Muir and M'Nee⁹ and Itami,¹⁰ following experimental hemolytic anemias.

⁹ Muir, R., and M'Nee, J. W., *J. Path. and Bacteriol.*, 1911-12, xvi, 410.

¹⁰ Itami, *Arch. exp. Path. u. Pharm.*, 1910, lxii, 104.



TEXT-FIG. 4.



TEXT-FIG. 5.

Increased Bone Marrow Activity During Subsidence of the Plethora.

The beginning hemoglobin drop in those rabbits which developed an anemia during or after transfusion was marked in each case by a prompt rise in the number of reticulated cells. This increase was rapid, and in two cases the number had risen above normal by the time the hemoglobin had descended to its original level (Text-fig. 4). Then, as the hemoglobin fell further and anemia developed, the reticulated cells continued to increase with great rapidity. The largest number was observed during the early period of regeneration. One rabbit showed a maximum of 594 per 1,000 at this time. They gradually diminished as the animal recovered from the anemia but were still increased when the hemoglobin reached normal again and remained fairly numerous for some time.

Attention is called to the fact that the number of reticulated cells began to increase soon after the hemoglobin started to fall from the plethora level and long before it had reached normal. A further study of this phenomenon was considered worth while. It seemed not unlikely that stimulation of the bone marrow at this time might be brought about by the greatly increased quantity of destroyed blood present. Accordingly an attempt was made to determine this possibility by injecting rabbits intravenously with laked blood. The rabbits were first rendered anemic¹¹ by bleeding, and the injections of blood were made at different stages of recovery. Some of the rabbits were treated when very anemic; others had practically reached normal before treatment was begun. In order to simulate as nearly as possible conditions of blood destruction occurring *in vivo*, the laked blood was injected at 1 hour or 2 hour intervals for periods of 1 to 3 days. Relatively large quantities were given without apparent ill effect. At no time during the course of the experiment was any increase in the number of reticulated cells noted, nor was other evidence obtained of increased bone marrow activity.

It was then found that simple blood removal from a plethoric animal by bleeding was sufficient to cause a marked bone marrow

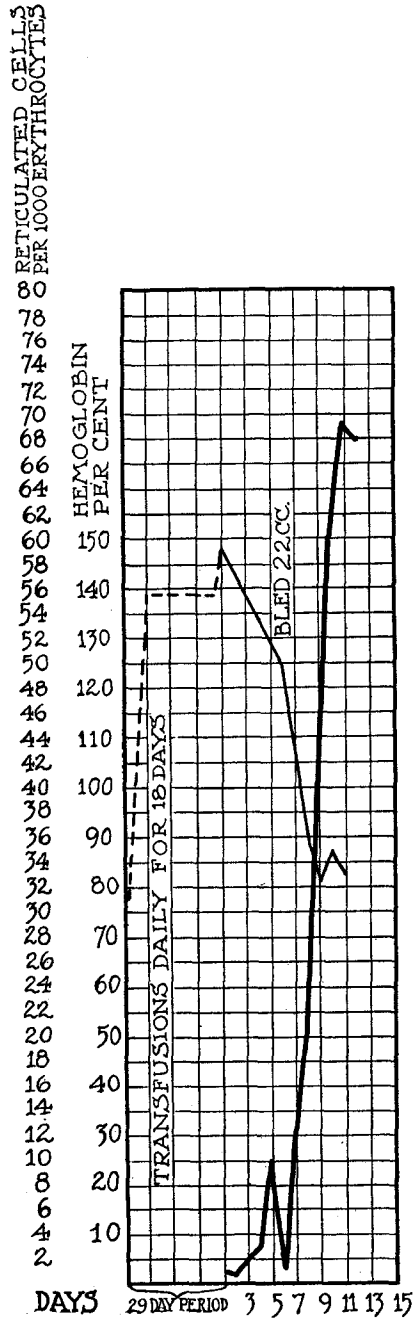
¹¹ The reason for producing a preliminary anemia in these rabbits is that the experiment was performed originally in an attempt to explain on experimental grounds the cause of the remission in pernicious anemia.

stimulation. Rabbits were made plethoric in the usual way and kept in this condition until the reticulated cells were much diminished. They were then bled a quantity calculated to bring their hemoglobin per cent back to almost normal. An immediate rise in the number of reticulated cells resulted. The normal number, it will be recalled, is 5 to 20 per 1,000 red cells. In one animal treated as above described, they increased to 69 per 1,000 (Text-fig. 6); in another to 80. A third rabbit showed even a greater increase, 108 per 1,000 (Text-fig. 7), but unfortunately the hemoglobin in this case fell after bleeding to slightly below normal. With a fourth animal, the increase was definite, but less marked. In Text-fig. 6 it will be noted that the fall in hemoglobin following bleeding extended over a period of several days, and that the reticulated cells had increased markedly some time before the hemoglobin reached its normal level. Text-fig. 7 shows an instance in which the drop in hemoglobin as a result of bleeding was later followed by a rise above normal. This secondary rise in hemoglobin and the high reticulated cell count were maintained for some days.

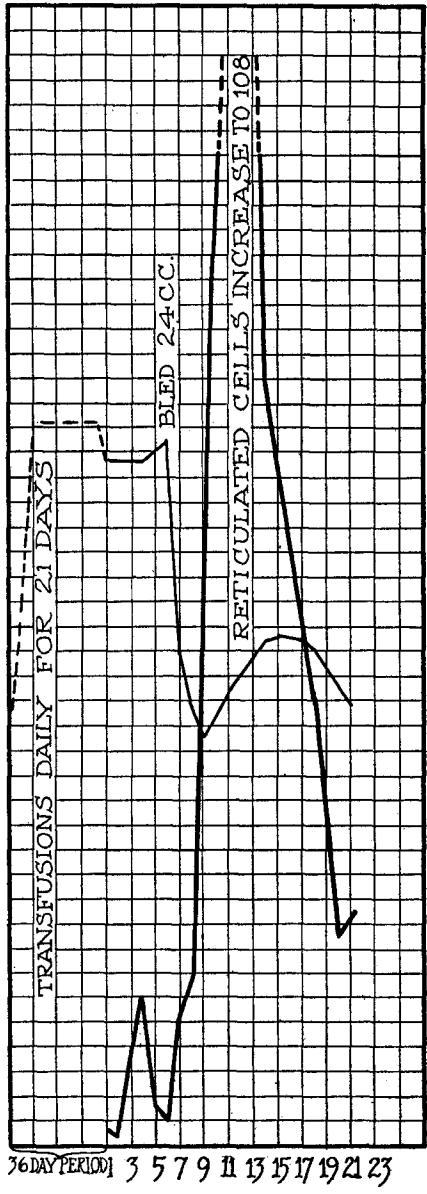
Although these experiments are not sufficiently complete to permit one to draw definite conclusions, yet the results would suggest that the increased bone marrow activity accompanying the initial drop of hemoglobin in the plethoric rabbits is due to some functional disturbance of the circulation, resulting from the rapid removal of the plethora. It seems not improbable that this may be a temporary relative oxygen deficiency explainable on the basis that during the period of plethora the organism had in some way adapted itself to a blood of greater oxygen-carrying power. Certainly the blood loss may be thought of as having resulted in a relative anemia.

Color Index.

Observations on the color index in those plethoric rabbits developing anemia revealed striking changes. The normal color index for rabbits is 0.65 to 0.75. During regeneration it was greatly increased, usually to 1.0 or even to 1.08. This finding is of interest particularly in its relation to the accompanying greatly increased percentage of reticulated cells in the blood. It would indicate that reticulation is



TEXT-FIG. 6.



not necessarily associated with a deficiency in the hemoglobin content of the cells and that this association in the blood of secondary anemia is largely fortuitous. In two of the present instances, the reticulated cells numbered 34 per cent and 60 per cent respectively of the total number of red cells, yet the color index was increased 50 per cent over the normal. Even allowing for the somewhat increased size of the reticulated cells, it does not seem possible that they contained less hemoglobin per unit of cell substance than the accompanying more normal red cells. In fresh films they were observed to be notably well colored.

Transfused Reticulated Cells.

In the work as thus far discussed, a possible source of error has not been considered; namely, that of the reticulated cells introduced with the transfused blood. The number of these may, and indeed sometimes must be, considerable, because the bone marrow of the animals furnishing the blood has been stimulated by repeated bleeding. After many transfusions one might expect the recipients to show high counts of reticulated cells. As a matter of fact the reverse is the case. What becomes of the reticulated cells introduced, whether they perhaps mature into non-reticulated corpuscles or instead are destroyed, remains to be determined. As bearing on the point, it should be mentioned that the microcytes of secondary anemia due to hemorrhage are largely derived from fragmentation of reticulated cells, and that in plethoric animals the fragmentation of red blood cells is much increased.⁷

No observations were made on the effect of plethora on the number of white cells or platelets.

Clinical Bearing.

The effect of experimental plethora on the bone marrow of rabbits has a direct bearing on certain unfavorable results which may occur after transfusions in human beings. Clinicians have observed that some cases of pernicious anemia receiving transfusion show no stimulation, but instead unmistakable signs of bone marrow depression.

Vogel and McCurdy⁶ were the first to report a systematic study of the reticulated cells in pernicious anemia patients with transfusion. In several of their cases, a decided drop in the per cent of reticulated cells occurred following transfusion. They attributed this to the diluting effect of the newly introduced blood, which had a much lower content of reticulated cells. These patients did badly.

At the Massachusetts General Hospital, Minot and Lee¹² have recently observed the effect of transfusion on the per cent of reticulated cells in a number of cases of pernicious anemia. In certain of their patients, a very marked diminution in the reticulated cells occurred following transfusion. This was accompanied by a leukopenia, a reduction in the number of platelets, and in some cases purpura as well, all of which went to form the picture of general bone marrow depression. As was to be expected, there was no increase in the red corpuscles after transfusion in these patients, other than that referable to the alien blood introduced. In their cases, the amount of blood transfused did not exceed 600 cc. and in one case it was only 300 cc. No such effects as those described above were observed after transfusions of less than 300 cc.

From the results described in the present paper, one may draw tentative conclusions as regards such instances. In pernicious, as in any form of anemia, oxygen deficiency resulting from blood loss doubtless constitutes the stimulus for increased blood production. It is conceivable that in severe conditions, a stage is reached where the bone marrow becomes so exhausted that there is danger of its failure to respond any longer to this stimulus; in other words, the stimulus of oxygen deficiency has grown relatively less effective and may at any time become insufficient. The introduction of a large quantity of blood into the circulation has inevitably the effect of reducing oxygen lack. The sudden lowering of stimulus thus brought about may result in a diminished activity of the bone marrow. The inference is clear that in pernicious anemia with a sluggish bone marrow as shown by the count of reticulated cells, small transfusions are preferable to large ones.

¹² Dr. Minot and Dr. Lee have very kindly allowed me to use these data, which they have not yet published.

SUMMARY.

With the purpose of determining whether a diminished activity of the bone marrow could be brought about experimentally, plethora was produced in rabbits by means of repeated small transfusions of blood. Counts of the number of reticulated red cells in the circulating blood were made during the course of the experiments as an index to changes in the activity of the bone marrow.

With the development of plethora, the number of reticulated cells in the blood decreased. In the majority of the plethoric animals, this diminution was extreme, and in some instances, reticulated cells practically disappeared from the blood. A comparison of the red bone marrow of these animals with that of normal controls revealed a marked reduction in the content of reticulated cells.

After a number of transfusions, there occurred in some of the plethoric rabbits a sudden and marked drop in hemoglobin. The hemoglobin continued to fall until a severe grade of anemia was reached. This was followed by an extremely rapid regeneration accompanied by a striking rise in color index. During regeneration, the reticulated cells were enormously increased in number.

Taken together, these facts show that the bone marrow is markedly influenced by plethora. The diminished number of reticulated cells observed, both in the circulating blood and in the marrow, would make it appear that a decided decrease in blood production occurs. The reduction in the number of these cells cannot be due to changes in the constitution of the red cells put out by the bone marrow, as a result of an increased quantity of hemoglobin in the body, because during regeneration from the above mentioned anemia, when the color index was very high, reticulated cells were still present in large numbers. That the activity of the bone marrow does actually diminish during plethora is further evidenced by the occurrence of the anemia. The most reasonable explanation of this phenomenon is that the recipient develops an immunity against the blood of the donors, which results in the destruction of the strange cells that are in circulation. In keeping with this conception is the appearance of isoagglutinins for the donors' red cells in the blood of the recipient, at about the time of the beginning fall in hemoglobin. The occurrence of anemia as a result of the destruction of the alien blood only would seem to be

due to the circumstance that, during the period of plethora, blood production is greatly diminished; as a consequence, the blood cells proper to the recipient are gradually reduced in number and replaced by alien cells until the latter come to constitute the bulk of the animal's blood.

In those rabbits developing anemia, the initial drop of hemoglobin from the plethoric level to the normal was constantly accompanied by a marked rise in the number of reticulated cells. This brought up a subsidiary problem for study. With the idea that the stimulation of the bone marrow might be due to the presence of an increased quantity of broken down blood, rabbits were injected intravenously with large amounts of laked blood cells. The procedure had no evident effect on the blood picture. It was then found that simple blood removal from a plethoric animal which brought back the hemoglobin to the normal level, or even to a point somewhat above, sufficed to cause a marked increase in the number of reticulated cells. Although these findings are not conclusive, they suggest an explanation for the increased bone marrow activity accompanying the initial drop of hemoglobin in the plethoric rabbits; namely, that the organism had in some way adapted itself during the period of plethora to the presence of a greater amount of blood and that the result of blood loss in such an organism was a relative but not absolute anemia.

The finding that the activity of the bone marrow can be depressed by the introduction of a large quantity of blood into the circulation accounts for the diminished bone marrow activity which sometimes occurs after transfusion in pernicious anemia. In such cases there is a marked drop in the number of reticulated cells and other evidence of bone marrow depression; the patient shows no benefit from transfusion or may grow rapidly worse. The cause of this depression is best explained on the basis that in severe instances of the disease where exhaustion of the bone marrow is imminent, the stimulus of the anemia is only just sufficient to keep the marrow functioning. A sudden lowering of this stimulus is brought about by the introduction of a large quantity of blood into the circulation, and the result is a fall in the activity of the bone marrow. It follows from this that in pernicious anemia with a feebly reacting bone marrow as indicated by the number of reticulated red cells, small transfusions are preferable to large ones.