Polycythemic Response in Normal Adult Rats to a Nonprotein Plasma Extract from Anemic Rabbits

By Henry Borsook, Ashton Graybiel, Geoffrey Keighley and Emanuel Windsor

The experiments reported here were suggested by the findings in two independent lines of investigation, one concerned with the erythropoietic response to high altitude1 and the other with the stimulating effect of liver and plasma extracts on the incorporation of amino acids in reticulocytes.2 Drabkins and Graybiel1 showed that in the acclimatization to low oxygen tension, heme (and inferentially hemoglobin) synthesis was accelerated. Among the several interpretations of this fact is the possibility that erythropoiesis is regulated by a humoral factor.3-5 It was also possible that the factor (or factors) in liver or plasma which stimulates amino acid incorporation (protein synthesis) in reticulocytes might be the humoral factor, or at least be related to it or function like it.

The factors found by Borsook and his associates6 to stimulate amino acid incorporation into rabbit reticulocyte proteins fall into three categories. One consists of certain amino acids: histidine, leucine, phenylalanine, tryptophane, and valine. A second they have designated as carbohydrate metabolism factors; these are glucose plus either adenosinetriphosphate, diphosphopyridine nucleotide, or triphosphopyridine nucleotide. Deproteinized extracts of liver and plasma are in a third class since their effects could not be ascribed to either amino acids, glucose, or any of the known cofactors, vitamins, or common metabolites they contain.

Inasmuch as it seemed unlikely that in normal animals feeding or injection of glucose and cofactors could have any significant effect, we tested the effects of increasing the amounts of the stimulating amino acids in the diet and of injecting extracts of liver and of plasma. Accordingly, the following amino acid supplement to the normal ration was prepared. To 265 Gm. of dry chow powder was added 0.7 Gm. of L-tryptophane and 2.8 Gm. each of L-histidine, L-leucine, L-lysine, L-phenylalanine, and L-valine. Fifty mg. of supplemental amino acids per 100 Gm. of body weight in the form of the mixture was the only food given the animals at night so that they ate all of it. In the daytime the usual laboratory feed was available to them ad lib. The group consisted of five male and five female

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Opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the view or endorsement of the Navy Department. Reference may be made to this report in the same way as to published articles noting author, title, source, date, project number, and report number.
normal adult rats. At the end of six weeks there was no significant change or trend in the amount of hemoglobin or the erythrocyte count of any of the animals.

Another preliminary experiment was conducted, with negative results, utilizing a deproteinized liver extract. However, positive results were obtained with deproteinized plasma, and these experiments will now be described.

**FIRST SERIES**

**Procedure**

The plasma extract was prepared as follows. One ml. of 2.5 per cent solution of phenylhydrazine hydrochloride (neutralized) was injected subcutaneously into adult rabbits. After seven daily injections the animals were anemic and 85 to 95 per cent of the erythrocytes were reticulocytes. Blood was collected by bleeding from the ear vein or by cardiac puncture. Coagulation was prevented by heparin, 20 mg. of sodium heparin per 100 ml. of blood. After centrifugation the plasma was pipetted off, the pH brought to 5.5 with HCl, and then it was boiled for 15 minutes. A volume of redistilled water equal to that of the plasma was added to the coagulum, stirred thoroughly, boiled for 5 minutes, then filtered. There were three such washings of the coagulum. The washings and original filtrate were concentrated in vacuo (internal temperature 35 to 40 C.) to the original volume of the plasma. This solution still contained a small amount of protein (denatured) and was usually strongly opalescent.

It was decided to test the extract on healthy rather than on anemic animals. This constituted a far more rigid test inasmuch as an increase in erythropoiesis would indicate that the normal balance between red cell formation and destruction had been altered. Sprague-Dawley rats were used. Experimental and control groups were litter mates matched for age, sex, weight, hemoglobin, and red cell concentration. The animals were 3 to 6 months old at the beginning of the experiment and their weight ranged from 250 to 450 Gm. Two ml. of the extract per 100 Gm. of body weight were injected subcutaneously daily. The animals ate normally, did not lose weight, and appeared healthy after four weeks of such injections.

In these experiments, strictly comparable control groups (see below) received daily injections of 2 ml. per 100 Gm. of body weight of Krebs’ solution (an isotonic solution of the inorganic salts in plasma) containing 100 mg. per cent glucose.

Blood was collected twice weekly for measurement of hemoglobin and hematocrit volume. To draw the blood the rat was wrapped in a towel with its tail protruding; the tail was nicked with a razor blade so that it bled freely. Blood was drawn up directly into the measuring pipets. The wound closed spontaneously.

The hemoglobin determination was a modification of that of Drabkin and Austin. To 0.02 ml. of blood was added 4 ml. of water. One drop of 2 per cent K$_3$Fe(CN)$_6$ was added to the hemolyzed blood and allowed to stand for 10 minutes. Then 0.5 ml. of 0.1 per cent KCN was added and diluted to 10 ml. with water. The photometric reading was made at 546 mp against a reagent blank. The standard was a pure cyanmethemoglobin solution treated as above. The hemoglobin concentration in the standard was computed on the basis that its nitrogen content was 16.7 per cent. The results are expressed as Gm./100 ml. blood.

The hematocrit was measured by a modification of the method of Van Allen. Blood was drawn up into a Van Allen pipet to the top of the scale, the outside of the tube wiped clean 1.8 per cent potassium oxalate was drawn up half filling the bulb, and the ends closed tight with a rubber band. The pipet was centrifuged at 825 g for 15 minutes, then turned through 180 degrees and centrifuged another 15 minutes. The volume of packed cells was read directly as per cent.

Plasma volume was determined by a modification of the method of Wang and Hegsted. The animal was brought under light ether anesthesia, the front of the neck shaved and washed with alcohol. A 2 cm. incision was made from the clavicle to the mandible, the jugular vein exposed by blunt dissection and injected with 0.4 ml. of 0.1 per cent Evan's
TABLE 1.—Mean Values of Blood Measurements on Female and Male Rats  
(In the experimental subgroups the animals received daily injections of plasma factor)

<table>
<thead>
<tr>
<th></th>
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<th>Weeks</th>
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<td>+1</td>
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<td>15.4</td>
<td>16.5</td>
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<tr>
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</tr>
<tr>
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<td>15</td>
<td>46.5</td>
<td>51.3</td>
<td>56.4</td>
</tr>
</tbody>
</table>

* Average of 4.  
† Average of 12.  
‡ Average of 10.  
§ Average of 9.

MALES  
FEMALES

LEGEND

- - - - - - Control
○ ○ ○ ○ ○ ○ Experimental

FIG. 1.—Changing pattern in blood of rats before and during the administration of the plasma factor. Each point represents the mean value based on the information contained in table 1.
TABLE 2.—Blood Volume of Experimental and Control Animals Twenty-seven Days after Beginning of Injections

(Each figure represents the mean of measurements made on four animals except for the experimental males which numbered three)

<table>
<thead>
<tr>
<th></th>
<th>Weight</th>
<th>Hb. (Gm./100 ml. blood)</th>
<th>Ht. (%)</th>
<th>Blood volume (ml./100 Gm. body weight)</th>
<th>Hb × B. V.</th>
<th>Ht. × B. V.</th>
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<td>14.0</td>
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<tr>
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<td>56.6</td>
<td>7.06</td>
<td>120</td>
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</table>

Blue in 0.9 per cent sodium chloride. Five minutes later 0.5 ml. of blood was withdrawn by cardiac puncture into a syringe moistened with a heparin solution. To 0.4 ml. of blood so drawn was added 3.6 ml. of 0.9 per cent sodium chloride mixed gently and the suspension then centrifuged 15 minutes. The color of the supernatant solution was measured against plasma diluted 10 times as a blank. The photometric reading was made at 620 μm and compared with a standard of Evan's Blue (10 μg. per ml. in 0.9 per cent sodium chloride). The results were expressed as ml. per 100 Gm. of body weight.

Results

The changes observed in hemoglobin and hematocrit values are summarized in table 1 and figure 1. No significant changes were noted until one week after beginning the daily injections of plasma filtrate. Then, both the hemoglobin and hematocrit values increased proportionally in the experimental animals; the males responding more slowly than the females. In the control animals there were no statistically significant changes throughout the experimental period.

The possibility was tested that the increases in hemoglobin and hematocrit volume in the animals receiving the plasma filtrate might be due to hemoconcentration. Plasma volume was determined on experimental and control groups of males and females approximately four weeks after the beginning of the injections. The results summarized in table 2 show that the blood volume was not significantly different in the experimental than in the control groups and that, if anything, the experimental animals had a somewhat larger blood volume. Since the hemoglobin and hematocrit values are concentrations, larger blood volume tended to reduce these values. It is clear that the increases observed in the experimental groups could not have been an expression of hemoconcentration.

SECOND SERIES

An independent assay of the plasma filtrate was carried out at the U. S. Naval School of Aviation Medicine in Pensacola, Florida.*

Procedure

One assay was carried out on male and the other on female rats of the Sprague-Dawley strain. There were four pairs of animals in each series. Each pair were litter mates, one serving as the experimental animal and the other as the control. In the males, the experi-

* We are indebted to Mr. James Colehour, head of the clinical laboratory, for his cooperation and to Miss Mary McPhaul and James Herbert Wagner, SN, USNR for technical assistance.
mental subgroup received daily injections of plasma filtrate for seven weeks and the control subgroup received Krebs' solution containing 100 mg. per cent glucose. In each instance the amount was 5.0 ml. injected subcutaneously. Measurements on the blood were made at intervals before, during, and after the period of injections. The procedure in the case of the females was the same except that they were sacrificed after receiving injections for one month for the purpose of studying the bone marrow.

Two days were usually required to carry out the blood measurements on each group, but for statistical purposes the figures were lumped. The determinations were carried out on about 0.3 ml. of heparinized blood. This was obtained by amputating a wafer-thin section of the rat's tail with an especially designed guillotine. The methods described in the first series were used in measuring the hemoglobin and hematocrit. The reticulocytes were stained and counterstained respectively with Azure II and Wright's stain.

**Table 3.—Mean Values of Blood Measurements on Female and Male Rats**

(The N in each subgroup is 4. In the experimental subgroups the females received daily injections of plasma factor during +1 through +4 weeks; the males during +1 through +7 weeks)

<table>
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<th>Weeks</th>
<th>−3</th>
<th>−2</th>
<th>−1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>+5</th>
<th>+7</th>
<th>+9</th>
<th>+12</th>
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<td>Hgb. (Gm. %)</td>
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<td>Female</td>
<td>Control</td>
<td>15.6</td>
<td>15.9</td>
<td>15.3</td>
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<td>14.9</td>
<td>14.4*</td>
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<td>RBC (million per cu. mm.)</td>
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<td>9.79</td>
<td>8.97</td>
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<td>8.67</td>
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<td>62.0*</td>
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<td>14.3</td>
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<td>15.0*</td>
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</table>

* This denotes a mean of three values. All others are means of four.
RESULTS

The chief findings are summarized in table 3 and graphically illustrated in figure 2. The results were clear-cut, both in the male and female animals. With the exception of the initial reticulocyte counts, the values for the control animals in the male group were quite uniform throughout the fourteen week period. The failure of these values to increase in young growing rats may have been due to blood loss. In contrast to the controls, the values in the case of the experimental
animals showed a rise shortly after the administration of the plasma extract and a fall to control level shortly after the injections were discontinued. Although the magnitude of the increase was not great it was significant, partly because of the temporal relationships which suggest cause and effect and partly because the increase was over and above normal values.

In the females, the magnitude of the response was greater than in the males. The spread between control and experimental values was exaggerated because the blood loss produced a noticeable effect in the control animals.

At the time the female rats were sacrificed, the marrow from the femurs was removed and smears prepared which were stained with May-Grünwald stain. The granulocytic series was counted as one class and the erythroid elements in three classes. The results, summarized in table 4, reveal insignificant differences between the control and experimental subgroups.

**DISCUSSION**

The important question is whether our results can be accepted as proof of the presence of an erythropoietic factor in the plasma filtrate. The evidence although indirect otherwise fulfills rigid criteria of erythropoietic activity. The increase in concentration of erythrocytes and hemoglobin in the peripheral blood can only be explained on the basis of hemoconcentration, mobilization of erythrocytes, or increased production. Hemoconcentration was ruled out in the series of experiments where plasma volumes were measured; indeed, the findings suggested that, if anything, the experimental animals had a larger blood volume than the control animals. Mobilization of erythrocytes from spleen or other organs is an unlikely explanation because of (1) the magnitude and long persistence of the increase and (2) the absence of hemoconcentration.

That increased production of erythrocytes and hemoglobin occurred is suggested not only by the increase in concentration of these elements in the peripheral blood, but also by the increase in reticulocytes and by the over-all pattern of the changes in the blood. Reticulocytosis was regularly observed. In series II the increase in reticulocytes was nearly as great in the control as in the experimental animals, probably because of blood loss. This may account for the failure

*We are indebted to Lt. JG. James H. Berrian, MSC, USN for this study on the bone marrow.*
to observe any difference in erythropoietic activity in the marrow between the control and experimental female rats. The most convincing evidence that the plasma filtrate caused reticulocytosis is seen in the notable decrease in number when injections were discontinued in the male experimental subgroup (fig. 2).

We were impressed with the remarkable similarity between the blood changes observed in these animals and in animals subjected to reduced oxygen tensions. As far as we are aware, this is the first proof that a tissue extract can stimulate erythropoiesis in normal animals comparable to that induced by hypoxia. The experiments of Erslev and Reissmann are of interest in this connection. The former observed an erythropoietic response in normal rabbits when injected with large amounts of plasma from anemic animals. Reissmann observed erythropoiesis in a parabiotic rat breathing normal air while the partner breathed a low oxygen gas mixture. Both of these experiments as well as our own strongly suggest that a humoral factor is at work.

It is interesting that the stimulating factor (or factors) was not destroyed by fairly prolonged boiling (15 min.) at pH 5.5 under which conditions nearly all the protein in plasma was coagulated. Some protein—material precipitable by 7 per cent trichloroacetic acid—remains in solution. As we have no information at present on the chemical nature of the stimulator, it would be premature to exclude the uncoagulated protein as the factor. The factor stimulating amino acid incorporation remains in solution in 7 per cent trichloroacetic acid. If the erythropoietic factor behaves similarly it is certainly not a protein, as conventionally defined. Whether or not these two factors are the same or related is under investigation.

It is not to be inferred that a similar result could not have been obtained with plasma extracts of animals with normal blood. The factor stimulating amino acid incorporation into rabbit reticulocyte proteins is present in the blood of all normal animals tested for this factor, in their erythrocytes, and in rabbit reticulocytes. Investigations are now planned to determine whether the erythropoietic factor is present in the plasma and red cells of normal blood of different animals. The isolation of the erythropoietic factor is in progress.

Initial experiments using extracts of plasma obtained from normal animals were negative, i.e., an erythropoietic response was not observed.

**Summary**

Experiments are described in which a protein-free extract of plasma obtained from anemic rabbits was injected into healthy young rats. The resulting erythropoietic response was comparable to that induced by hypoxia. The findings suggest that a humoral factor was at work which was capable of disturbing the physiologic mechanisms which establish the equilibrium between red blood cell formation and destruction. The far-reaching significance of these findings is apparent.

**Summario in Interlingua**

Es deschriebite experimentos in que un nonproteinic extracto de plasma ab conilios anemic eseva injicite in san e juvenile rattos. Le resultante responsa erythropoietic eseva comparabile a illo que es inducite per hypoxia. De iste
constationes on conclude que un factor humoral esseva involvite, le qual esseva capace a disordinar le mechanismos physiologic que establi le equilibrio inter le formation e le destruction del erythrocytos. Le implicationes extense de iste constationes es evidente.

REFERENCES
Polycythemic Response in Normal Adult Rats to a Nonprotein Plasma Extract from Anemic Rabbits

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