THE RELATION OF IRON AND COPPER TO THE RETICULOCYTE RESPONSE IN ANEMIC RATS*

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The phenomenon of reticulosis has been used extensively as a clinical indication of the activity of the hematopoietic organs since 1898 when Poggi (1) observed a rapid increase in the number of reticulocytes during blood regeneration in cases of anemia and a subsequent decrease when the erythrocyte count approached normal values. The rate of reticulocyte response has been found to be of distinct value in pernicious anemia studies. In 1928 Minot, Murphy, and Stetson (2) observed a response of reticulocytes, similar to that obtained in pernicious anemia, in cases of secondary anemia treated with liver. More recently Minot and his associates (3, 4), and Yang and Keefer (5) found a positive reticulocyte response in human cases of secondary anemia treated with iron. Although the factors affecting the production of reticulocytes during the treatment of pernicious anemia in humans have been investigated quite thoroughly, the mechanism of reticulocyte response during the treatment of secondary anemia has received little attention.

Very little is known about the reticulocyte changes in the blood of rats recovering from nutritional anemia. Much valuable information should be gained from studies with the rat because the requirements for hemoglobin formation are so well established. Beard, Baker, and Myers (6) found that iron alone, when fed to anemic rats subsisting on a milk diet, stimulated reticulocyte

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formation. When elements such as Cu, Mn, As, Ge, etc. were fed together with iron they found no greater degree of reticulosis but the rate of maturation of the erythrocytes was increased. Since the workers in Myers laboratory (7) have always obtained hemoglobin regeneration with iron alone, it is difficult to ascertain the action of copper from their work. The following studies were inaugurated to determine the exact relation of iron and copper to reticulocyte response in carefully controlled anemic rats.

EXPERIMENTAL

All animals were made anemic by the usual procedure (8). Supplements were not administered until a very severe degree of anemia was attained (2 to 3 gm. of Hb per 100 cc.). The supplements were in most cases given in the milk which constituted the basal diet. Hemoglobin determinations as well as erythrocyte and reticulocyte counts were made daily. For the latter purpose the method of Friedländer and Wiedemer (9) proved to be satisfactory in spite of its limitations (10). The reticulocytes were recorded in absolute numbers per c. mm. of blood.

The reticulocyte content of the blood of anemic rats shows some variation; the values, however, are seldom above 500,000 per c. mm. and generally fall between 200,000 and 400,000.

Chart 1 shows the daily changes in the hemoglobin, erythrocyte, and reticulocyte content of the blood of an anemic rat given 1 mg. of Fe and 0.1 mg. of Cu daily. Under optimum conditions for recovery from nutritional anemia, i.e. when adequate amounts of both iron and copper are fed, the increase in the number of reticulocytes is very rapid. A maximum of 1,500,000 to 1,700,000 per c. mm. is attained in 4 to 5 days after the mineral additions are started. This increase is followed by an almost equally rapid decrease to values between 200,000 to 400,000. In the meantime the hemoglobin and erythrocytes show a rapid and continued increase. The hemoglobin reaches values of approximately 8 gm. per 100 cc. before the number of reticulocytes begins to decrease. This response may be considered typical for our experimental conditions because similar results have been obtained with a large number of animals.

When iron alone was fed to anemic rats, the reticulocytes, erythrocytes, and hemoglobin showed no appreciable change.
The results for one of the rats fed iron alone are given in Chart 2. After this rat had received 0.5 mg. of iron daily for 29 days, 0.1 mg. of Cu was substituted for the iron. As soon as copper was supplied a response very similar to that shown in Chart 1 was observed. The increase in hemoglobin was not maintained in this case because the store of iron was soon depleted. These results clearly demonstrate that iron alone under the conditions of our experiments cannot stimulate reticulocyte formation. When a supply of both iron and copper is available in the animal the typical response occurs.

The effect of feeding copper alone is illustrated in Chart 3. A definite reticulocyte response was observed in all anemic rats given 0.1 mg. of Cu daily. The shape of the reticulocyte curve, however, is distinctly different from that observed in the case of a
Chart 2. Blood changes in a rat receiving iron alone. After 29 days of iron therapy copper was fed instead of the iron.
CHART 3. Blood changes in a rat receiving copper alone. The copper was replaced by iron alone after the rat had been on experiment for 49 days.
typical response. It is characterized by daily variations in the count as well as by a prolonged persistence of the reticulocyte values above the limits observed in untreated anemic animals. In the case of Rat 657 the higher level was maintained for 49 days as long as copper alone was supplied, but as soon as 0.5 mg. of Fe was fed in place of the copper a typical response was obtained and the level returned to normal. It is evident from the curves in Chart 3 that there occurred a slight initial rise of hemoglobin and erythrocytes with copper alone. This suggests that even severely anemic rats contain a small amount of iron which may be used for hemoglobin building in the presence of adequate amounts of copper. The very small increase in hemoglobin demonstrates that the anemic rats used in our laboratory are extremely depleted in iron reserves.

The effect of smaller amounts of copper fed with sufficient amounts of iron (0.5 mg.) is shown in Chart 4. Typical responses were obtained with levels down to 0.005 mg. of Cu. At this level the duration of the reticulosis was somewhat prolonged and the rate of hemoglobin and erythrocyte regeneration was much slower. With 0.001 mg. of Cu the hemoglobin formation was retarded and the reticulocyte response was much prolonged.

The results with feeding of smaller amounts of copper were more variable, probably owing to differences in the degree of copper depletion in the rats before the mineral supplements were added. Chart 5 shows the results when different levels were used for the same animal. In this case a slight temporary response was observed with 0.0005 mg. of Cu plus 0.5 mg. of Fe, but this response was undoubtedly due to a supplementary effect of small amounts of copper present in the animal. This reserve was soon used up because the hemoglobin and erythrocytes failed to increase appreciably. After the depletion period the addition of 0.001 mg. of Cu produced only a small response. When the copper intake was increased to 0.005 mg. the response was practically identical with that shown for this amount of copper in Chart 4. It is difficult to fix a definite minimum level of copper, which in the presence of sufficient iron will produce a typical response, but our results indicate that the level lies between 0.005 mg. and 0.01 mg. daily.

The curves in Chart 6 indicate in a similar manner that in the
**Chart 4.** The effect of different levels of copper on the reticulocyte response in the presence of adequate amounts of iron.
Chart 5. The effect of suboptimal amounts of copper on the reticulocyte response in the presence of adequate amounts of iron.
presence of adequate quantities of copper the anemic rat requires at least 0.3 mg. of iron daily for the production of a typical reticulocyte response. The response in the presence of 0.2 or 0.1 mg. of iron was more prolonged. The results for the rat receiving only 0.04 mg. of Fe are very similar to those obtained with many of the animals fed copper alone. When some response is obtained with such minute quantities of iron it is easy to see why a response may be observed with copper alone. Even the most anemic animal may contain iron which can be mobilized for hemoglobin formation in the presence of copper.

The fact that both iron and copper are necessary to produce marked reticulosis in anemic rats, and that these elements are also essential for hemoglobin formation suggests an intimate relationship between the two processes. In order to study this relationship further the effect of hemoglobin injections on the reticulocyte content of the blood of anemic rats was studied. Since it is very difficult to prepare any quantity of pure undenatured hemoglobin, blood preparations were made according to the method of Whipple and Robscheit-Robbins (11). The hemoglobin solutions were prepared from rat blood and 2 cc. containing 0.08 gm. of hemoglobin were injected intraperitoneally into anemic rats every other day. No ill effects from the injections were observed in any case.

The blood changes for one of the four rats that received this treatment are given in Chart 7. It is readily seen that the reticulocyte response is very similar to that obtained when suboptimal amounts of iron and copper were fed. The reticulocytes remained at a high level as long as the injections were continued and the hemoglobin and erythrocytes increased very slowly. These results indicate that the presence of added hemoglobin in the blood was sufficient to stimulate reticulosis but the quantity was not large enough to build up the hemoglobin content of the blood and consequently the reticulocytes remained at a high value. It is difficult to establish definitely whether the active substance is actually hemoglobin or some other constituent of the blood preparation. Oka (12) working with rabbits suffering from posthemorrhagic anemia reports a similar conclusion.

Through the work of Minot and his associates it is definitely established that liver preparations which are active in the cure of
Chart 6. Reticulocyte response with varying amounts of iron in the presence of adequate quantities of copper.
Chart 7. Blood changes during intraperitoneal injections of hemoglobin solutions.
pernicious anemia produce a reticulocyte response in humans. A large percentage of the cases of secondary anemia fail to respond to the feeding of pernicious anemia fractions. The question therefore arises: does a reticulocyte response occur without hemoglobin formation when secondary anemias are treated with pernicious anemia fractions or are these fractions unable to stimulate reticulosis in the absence of hemoglobin regeneration? If the suggestion that rapid hemoglobin formation initiates a reticulocyte response during the treatment of secondary anemia is correct, then a pernicious anemia fraction low in iron and copper should have no effect on the reticulocytes in the blood of anemic rats.

Several liver extract preparations used for the treatment of pernicious anemia were fed to anemic rats. Some were administered orally, and one preparation which was highly purified was injected intraperitoneally. The unpurified preparation produced a small but definite response; however, when the material was freed from all or most of the copper the response was very slight. This indicates that any stimulating effect which is obtained with liver extracts in this type of anemia is due mainly to the presence of iron and copper and that if these elements are removed liver preparations are unable to produce a typical reticulocyte response in secondary anemia.

**DISCUSSION**

From the results presented it is evident that iron alone is equally inadequate for the production of reticulocytes as it is for the regeneration of hemoglobin in rats depleted of their stores of copper and rigorously denied access to appreciable amounts of this element. Only when iron is supplemented by definite amounts of copper can a typical reticulocyte response be obtained. This is opposed to the results of Beard, Baker, and Myers (6) working with rats, and in apparent contradiction to the observations made on humans by Minot and Heath (4), Josephs (13), and others. However, the relatively small amounts of copper which have been shown to be essential for stimulation of hematopoietic activity furnish a basis on which this apparent disagreement of results can be explained. From Chart 4 it is evident that an intake of only 0.005 to 0.01 mg. of copper daily is necessary in the presence of sufficient iron to bring about a typical reticulocyte response,
and that still smaller amounts of copper may lead to increased numbers of reticulocytes. The fact that Beard, Baker, and Myers obtained a reticulocyte response with iron alone clearly demonstrates that their rats had some available copper with the iron. Whether the source of this copper was from the animal or from the milk we cannot say. Our results also indicate that more precautions must be taken to prevent reticulocyte response than are necessary to prevent appreciable hemoglobin regeneration when iron is fed alone. The rats used in our experiments ingested 0.007 to 0.008 mg. of Cu daily from the milk (50 cc. of milk containing 0.15 mg. of Cu per liter). It may be suggested that this amount of copper should produce a reticulocyte response since definite responses have been observed with 0.005 mg. of copper daily. The copper in the milk itself may not be as available as that added in the form of copper sulfate or that entering the milk from copper contamination.

We cannot agree with Beard, Baker, and Myers that copper acts by increasing the rate of maturation of the red blood cells. The decrease in reticulocyte concentration is not dependent upon copper per se but upon the amount of hemoglobin present in the blood.

Work with humans naturally cannot be controlled with the precision possible in animal experimentation. When iron alone is administered to humans suffering from secondary anemia, there is no way of estimating the store of available copper in the body, and it is impossible to attempt a depletion of this store before the iron is given. Anemic patients, therefore, may in many cases contain or ingest from the food sufficient copper to meet the requirements. Thus, it is easy to see why a typical reticulocyte response is believed to be dependent upon the addition of iron alone. The fact that an increase in hemoglobin is obtained in the human cases indicates that copper is available, for hemoglobin cannot be formed in the absence of this element. The complete mechanism of reticulosis can only be studied when carefully controlled animals are used.

Heath, Strauss, and Castle (14), suggest from their work with humans that there is a close correlation between the hemoglobin of the blood and reticulocyte response. We are in complete accord with this view. The rapid and large increases in reticulo-
cytes which we observed when optimum amounts of iron and copper were fed are undoubtedly due to the very low hemoglobin content in the blood of the rats used. A careful inspection of all our curves indicates that a slight increase in hemoglobin is generally observable whenever an increase in reticulocytes is obtained. This increase was not evident in every case but the amount of hemoglobin required may be so small that it is difficult to detect with the method used. There is also the possibility that the active material is not hemoglobin but a precursor which is dependent upon iron and copper for its formation. Cooke (15) has suggested that the stainable substance of reticulocytes is probably a hemoglobin compound. It is also evident from all the curves that the number of reticulocytes recedes to normal values only after the hemoglobin has reached values of about 8 gm. per 100 cc.

Cases of posthemorrhagic experimental anemia which show spontaneous remission form no exception to the work reported here. In these cases the elements necessary for hemoglobin formation are present in the animal and therefore reticulocyte response may be observed without ingestion of iron and copper.

The results obtained with liver preparations indicate that the active material in liver cannot produce reticulosis in all forms of anemia. In pernicious anemia the response is due to an organic factor present in liver, but in secondary anemias the response is dependent upon the ingestion of sufficient iron and copper for hemoglobin formation. It is very probable that even in pernicious anemia the reticulocyte response following liver therapy depends in part upon the presence of sufficient available iron and copper.

**SUMMARY AND CONCLUSIONS**

1. A typical reticulocyte response characterized by a very rapid increase in reticular red blood cells to a maximum of about 1,500,000 per c. mm. in 4 to 5 days and an almost equally rapid decrease to values between 200,000 to 400,000, together with a definite increase in hemoglobin and erythrocytes has been observed in a large number of severely anemic rats given adequate amounts of iron and copper.

2. Neither iron alone nor copper alone can produce a typical reticulocyte response in rats suffering from severe nutritional
anemia. Iron alone fails to initiate a response, while copper alone produces a small prolonged response. The results with copper alone are undoubtedly due to the action of the copper on small available supplies of iron in the body.

3. The minimum daily requirements for the production of a typical reticulocyte response in an anemic rat are approximately 0.3 mg. of iron and 0.005 to 0.01 mg. of copper.

4. The intraperitoneal injection of blood hemoglobin into anemic rats caused a reticulocyte response similar to that obtained with suboptimal amounts of iron and copper.

5. Pernicious anemia fractions low in iron and copper failed to produce a typical reticulocyte response.

6. The action of pernicious anemia factors and iron and copper in the treatment of pernicious and secondary anemias is discussed.

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