

## EFFECT OF CERTAIN TISSUE EXTRACTS ON RED BLOOD CELL REGENERATION.\*

BY ANDREW JENEY AND JAMES W. JOBLING.

*(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)*

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While most of the anemias in human beings are due to some well known cause, and are for this reason looked upon as secondary, there are instances in which the cause cannot be determined, though the blood changes indicate that even these are of the secondary type. For this reason most of the experimental work done in the past in the attempt to obtain more information as to the cause of anemia in human beings, particularly pernicious anemia, has been based on the supposition that it is due to some form of intoxication. Recently, however, evidence has been furnished indicating that the course of experimental anemias in animals, as well as of pernicious anemia in human beings, may be favorably influenced by special diets. This suggests that some of the obscure anemias may be due to the lack of some substance in the body which is essential to normal blood formation and which may be furnished in the food. The recent work showing that calcium metabolism may be affected by various means suggests that the same may be true of iron.

Experiments planned to show the effect diet may have in producing anemia in animals have not been very fruitful. Many such attempts have been made with diets poor in iron, or in some other supposed essential, but the results were usually negative. Malnutrition in animals can be easily produced, but this is never accompanied with the characteristic blood changes seen in pernicious anemia.

Usually it is thought that the oxygen requirement of the body is the most important controlling influence in determining the number of red blood cells in the circulating blood, and this belief has received

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considerable support from the experiments made on animals and human beings which had been kept at high altitudes. However, neither this work nor the work with diet proves that there may not be some other regulatory mechanism, possibly located in the liver.

In 1904 Perrin (1) reported good results in the treatment of three cases of severe anemia, which were associated with hepatic cirrhosis, by adding fresh liver to the diet. The results obtained with the feeding of liver by Whipple (2) and his associates in the treatment of experimental anemia in dogs and by Minot and Murphy (3) in the treatment of pernicious anemia, have made it evident that this organ plays an important part in red blood cell formation. Recently Whipple and Robscheit-Robbins (4) have found that water and alcoholic extracts of liver fed to dogs with long continued anemia likewise promote red cell regeneration. It will be noted that all of Whipple's experiments were made with dogs which had a long continued anemia.

The belief has been prevalent for a number of years that the spleen in the adult, through a stimulating action on the bone marrow, influences blood formation. Though this view is not accepted by all observers, it is well known that it is directly concerned in blood formation during fetal life, and particularly during adult life, in the processes of red blood cell destruction. But what is the evidence in support of the belief that the spleen produces an internal secretion which regulates the number of red blood cells in the circulation? For a detailed discussion of the functions of the spleen the reader is referred to the recent papers by Eddy (5) and Krumbhaar (6).

Brinckmann (7) found that feeding normal rabbits with fresh ox spleen caused a reduction in the red and white blood cells. Zalinski (8) and Danilewski (9) reported a marked increase in the number of red cells by a single intraperitoneal injection of splenic extract, but Patou, Gulland and Fowler (10) were unable to confirm these observations. Krumbhaar and Musser (11) also report an increase in the red count following the injection of a fresh splenic extract. This increase lasted 1 or 2 days. Eddy (5) in his experiment used saline extracts of powdered dried spleen and protein-free extracts of fresh and dried spleen. The red cell counts were made at half hour intervals and continued 4 to 5 hours after the injection. Each of his preparations caused a decrease in the number of red cells in the circulation. On the other hand, Leake and Bacon (12) report that the feeding of fresh spleen, or the intravenous injection into rabbits of saline solution extracts of fresh spleen, caused a fall in the number of circulating erythrocytes within 24 hours. It went to 7 per cent above normal after the 3rd day, but soon fell to normal when administration was discontinued.

#### *Methods.*

Extracts of liver, spleen, gastric mucosæ and hemoglobin solution were used in the experiments. The gastric mucosæ were obtained from pig stomachs, and the

other organs from rabbits or calves. For preparing the protein-free extracts the tissues were ground to a pulp by passing repeatedly through a meat grinder. They were then extracted with an equal volume of 95 per cent alcohol to which had been added 0.5 per cent acetic acid. After standing overnight in the ice box the mixture was passed first through gauze and then through filter paper until the filtrates became clear. The tissue remaining was extracted twice in the same manner with 75 per cent alcohol. The filtrates from the three extractions were combined and evaporated to dryness at a temperature not exceeding 45°C., under negative pressure. The partially dried mass was then extracted with 75 per cent alcohol, filtered and the filtrate again evaporated to dryness. For one experiment, Dr. Zucker, of this department, kindly prepared for us a fat-soluble non-saponifiable extract of the liver.

In addition to the above, simple saline extracts of the tissues were used. These extracts were prepared by grinding up the fresh organ with saline solution in a mortar. Ten parts of saline solution were used to 1 of the tissue. The mixture was kept in the ice box overnight and then cleared either by filtration through sterile filter paper or by centrifugalization. The hemoglobin solution was made by laking 15 parts of washed rabbit red cells with 25 parts distilled water and cleared by centrifugalization.

Rabbits were used in all experiments. They were made anemic by bleeding from the heart; ether anesthesia was used. If necessary to save the life of the animal, the blood lost was replaced with saline solution. The blood removed constituted about 3 per cent of the body weight, or about 60 per cent of the total volume. Several blood counts, hemoglobin determinations and reticulated cell counts, were made before the bleeding and at frequent intervals afterwards. At the end of each experiment autopsies were done, particular attention being paid to the organs concerned in blood destruction and regeneration. The details of the autopsy findings will be reported subsequently.

The normal red count in our rabbits was found to be between 5 millions and 6 millions, which the bleeding usually lowered to about 2.5 to 3 millions. Only those animals with low red cell counts were used in the experiments. The animals were all kept under the same conditions as to light, food and temperature, and were weighed regularly. Our interpretation of the results of the injection of the different substances was based upon the rapidity with which the normal count was restored, as compared to a series of similar untreated animals. Each of the experiments has been repeated several times during the last few years.

In our preliminary experiments we found that the red count in the control animals losing about 60 per cent of their total blood was usually

not restored to normal until about 3 to 4 weeks subsequent to the operation, though evidence of regeneration became quite evident at the end of the 2nd week. There was also a marked loss in weight during the first 2 weeks. With the increasing red count there was an increase in reticulocytes.

The first experiment was planned to show the effect of protein-free extracts of the liver, spleen and stomach on normal animals. Two rabbits were used for each extract. The freshly prepared extract in doses of 5 mg. per kilo of body weight was given subcutaneously on 6 successive days in each week. As no effect was apparent after the 1st week, the dose was then increased to 10 mg. and this continued for a period of 25 days.

The animals after receiving the liver extract showed some increase in red cells after 30 days, but the increase was so slight that it was within the limits of normal variations in the red cell count. There was an increase in the neutrophilic leucocytes. Those receiving the stomach extract showed a very slight decrease. On the other hand, the rabbits receiving the spleen extracts showed a decrease of 1.8 and 3 millions, respectively. The number of reticulocytes was increased in the animals receiving the liver extracts, but was not affected by extracts of spleen and stomach. There was some increase in the lymphocytes.

This experiment was repeated several times with the same results as regards the liver and stomach extracts. In subsequent experiments, however, the spleen extract, whether protein-free or from freshly prepared cell emulsions in salt solution, was more active in causing a decrease in the red cell count. In the animals receiving the spleen extracts the per cent of reticulocytes, regardless of the degree of anemia, was always low, sometimes considerably below normal. There were no changes in the white blood cells which could be ascribed to the extracts.

The preceding experiment was repeated with animals which had been made anemic by one large bleeding. 2 days subsequent to the bleedings the red cell counts in this series of animals were between 2.5 and 3.2 millions. Three were left as controls, while the others received 10 mg. doses of the extracts of liver, spleen and stomach. All injections were made into the subcutaneous tissues.

Blood regeneration in the animals receiving the liver extract appeared to be a little more active than in the controls, and the reticulocytes increased earlier and were somewhat more numerous. The neutrophil leucocytes also were somewhat increased. The extracts of the gastric mucosæ appeared to exert no influence on regeneration. In the animals receiving the spleen extract there was very little red cell formation. When small doses were given there was a prolonged period during which there was little evidence of regeneration; but with larger doses the red count was still further depressed. The number of reticulocytes in these animals was very low.

A new series of rabbits was made anemic by bleeding from the heart and then injected with extracts, both saline and protein-free, prepared from the liver and spleen of rabbits which had been made anemic by repeated bleedings. The animals from the organs of which the extracts were prepared were bled every other day for 18 days. During this period a little more than 200 cc. of blood was taken from each animal, so that the final red cell count in each was a little less than 2 millions, and the hemoglobin approximately 20 per cent. The average number of reticulocytes for the four animals in the beginning was .5 per cent; after 13 days 7 per cent, and after the final bleeding, approximately 21 per cent. The animals were bled to death under ether anesthesia and the organs used for the preparation of extracts. Protein-free extracts were prepared from the livers of these animals. Because of their small size we were compelled to rely on saline extracts of the spleens. From the blood obtained at the last bleedings, hemoglobin extracts were prepared. The methods used in preparing these extracts have been described.

The hemoglobin solution was injected every other day into the peritoneal cavity of the rabbits in doses of 10 cc. The spleen extract was given in doses of 2 cc. and the liver extract in doses of 10 mg. per kilo, both subcutaneously.

Again, in this experiment, the spleen extract rabbits showed practically no evidences of any ability to generate red cells. The reticulocytes were not increased above the normal, which suggests that the effect noted above may be due to an inhibiting action which the spleen extracts have on blood regeneration, rather than a destructive action, which we at first thought probable.

On the other hand, the rabbits receiving the hemoglobin and liver extracts did show active red cell regeneration by the end of the 2nd week, and the per cent of reticulocytes was somewhat higher than that seen in the controls for the corresponding period.

In the next experiment we tested the action of a fat-soluble non-

saponifiable liver extract kindly prepared for us by Dr. Zucker of this department. In addition, we again studied the action of the protein-free extracts of the liver and spleen. This spleen extract had been kept in the ice box for more than 5 months.

The results of this experiment indicated that the fat-soluble non-saponifiable liver fraction was inactive, while the spleen extract, in spite of its age, showed the usual inhibiting effect. The blood regeneration in the rabbits receiving the non-protein liver extract was quite active.

Though none of the extracts used appeared to be very active in stimulating blood regeneration, yet there was some indication that the normal red cell count was restored a little more quickly in the animals which had received the liver extract and hemoglobin solution than in the untreated anemic controls. As extracts prepared from the organs of animals made anemic by bleeding were inactive, our next experiment was made with extracts prepared from the organs of animals made anemic by destroying the red cells within the body. This was accomplished by giving intravenous injections of distilled water. The injections were given five times in 2 weeks. One animal received 140 the other 205 cc. of water. The purpose of the experiment was to see if the organs of animals made anemic in this manner would contain the supposedly stimulating end-products of the red cells. Briefly, the results of the injection of these extracts showed no difference from the preceding.

While preparing these animals we were surprised to note the amount of distilled water which could be given in this manner without causing death. In subsequent experiments it was found that rabbits would frequently survive doses of 80 cc. and 120 cc.

#### DISCUSSION.

In part of our experiments with liver extracts we obtained results which indicated that they contained active substances, but while red cell regeneration began early in all the liver extract animals, a few of the controls showed the same early regeneration. In addition, the differences were not striking in any instance and were within the limits of experimental error. For this reason they were repeated several times over a period of more than 3 years, with the results mentioned.

Of the different extracts used, those prepared from the livers of anemic animals acted best, as the red count in the rabbits receiving these preparations apparently was restored to normal more rapidly than in the controls, and the reticulocyte count was definitely higher.

On the other hand, the effect of the various spleen extracts was quite striking. Where the amounts given were small, the regeneration of blood was much slower than in the control animals; while with large doses there was a further decline in number of red cells. The reticulocytes were always much fewer in number. It is possible that we

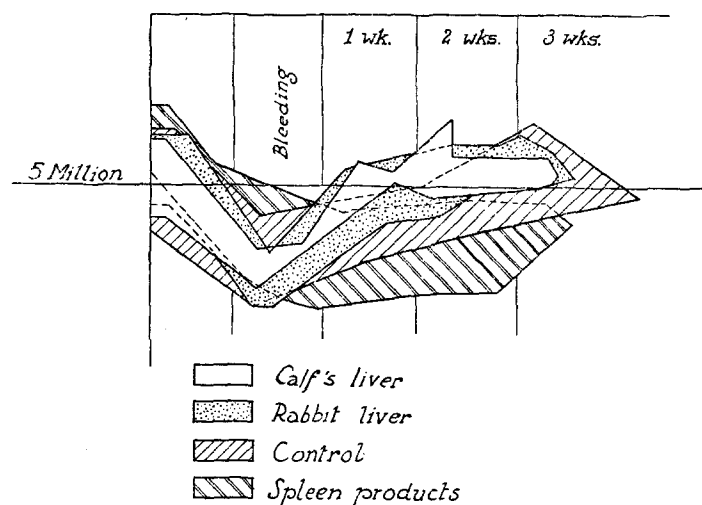


CHART 1.

were dealing with a poison, but the low reticulocyte count suggests inhibition of red cell formation rather than cell destruction.

In the body of the paper we have given no tables showing red cell counts, hemoglobin, etc. Had the results been more positive in character, they would have been included, but under the circumstances it seemed unnecessary to do so. The chart shows more graphically the results we obtained in our experiments with liver and spleen extracts, and represents the averages obtained in numerous experiments. It suggests that blood regeneration in the animals which received both rabbit and calf liver extracts is more active than in the controls, but

the difference is not marked. On the other hand, the spleen obviously has an inhibiting effect.

Our results are particularly interesting in view of those obtained with the feeding of liver by Whipple in the experimental anemias of dogs, and by Minot and Murphy in pernicious anemia. Several explanations of the discrepancies are possible. First, rabbits may not respond to the liver treatment; second, the active substance may have been destroyed by the heat used in drying our alcoholic extracts, though it did not exceed 45°C.; third, our preparations were given by subcutaneous injection, their's by mouth; fourth, our animals were made anemic by a single large bleeding, while Whipple's experiments were conducted with animals having a chronic anemia produced by repeated bleedings.

It seems not improbable that the marrow of animals with a long continued anemia will be hyperplastic and react more readily to the presence of a stimulating substance than those with an acute anemia produced by a single large bleeding. In the former case the marrow is prepared to act, in the latter case it is not.

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