HEMOGLOBIN REGENERATION IN THE CHRONIC HEMORRHAGIC ANEMIA OF DOGS (WHIPPLE)

I. The Effect of Iron and Protein Feeding

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The evaluation of the potency of a large variety of materials for hemoglobin regeneration in the chronic hemorrhagic anemia of dogs by Whipple and his associates (1) has stimulated further investigation of this condition. The less startling effect of liver feeding in the various types of human secondary anemia (2) raises an interesting question concerning the metabolism of iron and hemoglobin. Why, for example, is liver more effective in these anemic dogs than inorganic iron?

An analysis of the daily intake of calories, protein, iron and copper in the diets of 60 representative protocols taken from the publications of the Rochester laboratory, shows for the fruits, vegetables and dairy products, a close correlation between hemoglobin regeneration and the amount of iron ingested when the factor of the availability of these forms of food iron stressed by Elvehjem and his associates (3) is considered. With the glandular, however, and, to a less extent, the muscular foods other factors besides their iron content and its availability to the animal appear to be operating. Perhaps the chronic hemorrhage, in the removal of large amounts of blood constituents other than hemoglobin, and the unfavorable diet used in developing a slowly and constantly regenerating anemia, lead to a deficiency of materials besides iron involved directly or indirectly with hemoglobin formation. In such multiple deficiencies the addition of one of the materials which is lacking causes improvement in the animal's condition. This phenomenon may be considered as a type of mass action effect illustrated by the relationship of copper and iron in the milk anemia of young rats (4) or the influence of the relative amounts of calcium, phosphorus and iron present in the diet on the absorption of these materials (5) by the gastro-intestinal tract.

The standard basal salmon bread ration supplies a daily copper intake of 1.0 to 1.5 mg. A copper deficiency in these dogs is unlikely considering the small amounts of this element required for iron utilization by the anemic rat. Since liver is more effective than iron in these anemic dogs and the two together develop a summation response (6) our attention is turned to some other, possibly organic, factor present in the liver which may be effective in hemoglobin regeneration. The important observation (7) that the iron-poor liver of the horse made anemic by bleeding, loses none of its potency for hemoglobin regeneration in anemic dogs, stimulates this search for another factor in a multiple deficiency state. The rôle of a protein, namely casein, was studied as the first possibility.

Methods

A detailed description of the care of the dogs, the production and maintenance of the anemia and the experimental procedure is unnecessary since Whipple's methods have been duplicated to the best of our ability (8, 9). Briefly, dogs were fed the salmon bread ration developed in the Rochester laboratory and were bled from the femoral artery at the intervals and in the amounts necessary to produce, at the end of 8 to 12 weeks' time, an anemia with a hemoglobin level about one-third of the normal and a rate of regeneration not greater than 2 gm. of hemoglobin a week. Diets to be assayed were fed daily for 2 weeks. The hemoglobin producing power was measured by the amount of hemoglobin bled in order to restore the blood hemoglobin to its original level and to return the rate of regeneration to the basal of 2 gm. per week. After a special diet is discontinued, bleeding during a period of 2 or 3 weeks is usually required while the animal is eating the basal ration.

Hemoglobin was estimated with a Sahli-Leitz hemoglobinometer calibrated so that 14 gm. of hemoglobin equalled 100 per cent. These readings were checked with the blood iron method described by Hanzal (10). Red blood cell and white blood cell counts were performed with Bureau of Standards apparatus. Hematocrit and plasma volume determinations employed the methods described by Hooper, Smith, Belt and Whipple (11) using the brilliant vital red dye and 15 cc. graduated centrifuge tubes containing 2 cc. of 1.6 per cent sodium oxalate. Blood films were made on cover slips previously coated with brilliant cresyl blue. Blood for these determinations was obtained from the femoral instead of the jugular veins. All of these observations were made on each dog on a regular day each week. The daily food consumption was recorded.

The care necessary to maintain such anemic dogs in a constant condition for assaying the hemoglobin producing power of a material requires emphasis. Whipple has called attention to the effect of a preceding favorable diet on a subsequent hemoglobin response to another diet fed after an interval of many weeks. If other factors in the animal's condition besides the hemoglobin level such as the red blood cell count, the reticulocyte level, the white blood cell count, hematocrit, plasma volume, body weight, general appearance and activity, are considered carefully, this source of error can be eliminated.

The basal diet was restricted to the salmon bread developed by Whipple and Robscheit-Robbins (9) with the exception of Dog 4 which received in addition 430 gm. of fresh, whole, pasteurized milk daily. The daily diet was computed on the basis of 80 calories per kilo of body weight. Distilled water was kept in the cages at all times and was used in the preparation of the salmon bread. The cages were small enough to limit activity, and the dog room was well ventilated. An anthelmintic dose of tetrachlorethylene was administered routinely at 6 month intervals.

All of the diets were analyzed for their content of iron, copper and protein nitrogen. Iron was determined by the method employed by Elvehjem (12), the accuracy of which has been proved in a comparative study of several analytical procedures (13). The Biazzo method for copper analysis described by Elvehjem and Lindow (14) was used. Protein nitrogen was determined by macro Kjeldahl determinations of the difference between the total nitrogen and the nitrogen in a trichloracetic acid extract of the material. Caloric values were not determined; food table values were used (15). The foods employed had the following nutritional properties based on the weight as fed: salmon bread, 4 calories per gm., 9 per cent protein, 3 mg. per cent iron, 0.4 mg. per cent copper; milk, 0.7 calories per gm., 3.3 per cent protein, 0.05 mg. per cent iron, 0.015 mg. per cent copper; casein (Lister), 3.4 calories per gm., 84.5 per cent protein, 4.3 mg. per cent iron, 0.4 mg. per cent copper; frozen beef liver, 1.3 calories per gm., 22.5 per cent protein, 5 mg. per cent iron, 2.6 mg. per cent copper. Iron was added to the dog's ration in the form of a solution of ferric ammonium citrate in dilute hydrochloric acid with an iron concentration of 10 mg. per cc.

With these constituents, additions to the basal ration were planned whereby diets of liver or of casein and inorganic iron were obtained in which the animal received the same amounts of iron and protein. The hemoglobin response to these diets which quantitatively contained equal amounts of iron and protein of different sources, was compared with the response to the addition of an equal amount of inorganic iron without any additional protein. Calculations based on surface area instead of body weight have yielded no further information.

The weekly basal rate of hemoglobin production on the salmon bread diet was determined for each dog during 8 week periods and was found to be as follows: Dog 1, 1.8 gm.; Dog 2, 2.5 gm.; Dog 3, 1.7 gm.; Dog 4, 2.2 gm. This gave an average of 2 gm. a week arising from the salmon bread ration. In order to compare the effect of additions to the basal ration, this control rate of regeneration has been subtracted from the total hemoglobin removed by bleeding during the experimental feeding periods. The analysis of Whipple's protocols mentioned above shows that hemoglobin production is correlated with the additional iron but not with the total iron of the diet period.

RESULTS

Chart 1 shows the results obtained with two of the four dogs studied. The addition of milk to the basal diet of Dog 4 was originally planned

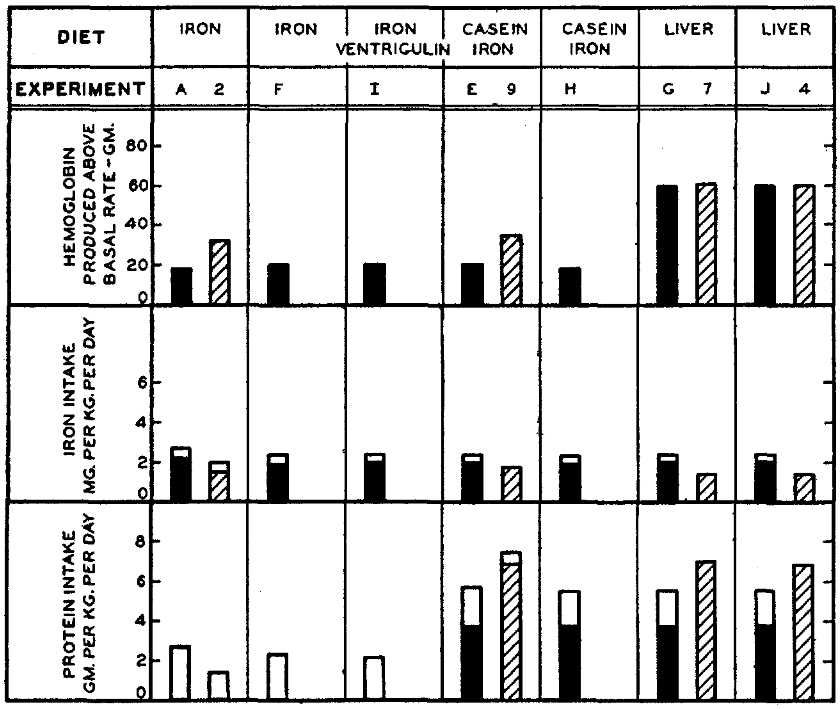

CHART 1. Hemoglobin produced above the basal rate in response to the addition to the diet of amounts of iron, casein-iron and liver during 2 week periods, representing equivalent quantities of iron and protein nitrogen. The open areas represent the iron or protein intake arising from the salmon bread and milk component of the diet. The numbered and cross-hatched experiments are on Dog 1, the lettered and full block ones on Dog 4.

as a check on the possibility of a protein (casein) deficiency in the salmon bread which contains only 9 per cent protein of fish and cereal origin. The results were, however, the same for all of these dogs. Seven experiments on Dog 4 and four experiments on Dog 1 are shown in the chart. Dogs 2 and 3 showed similar responses. Dog 4 also

TABLE I

The Response of Dog 4 to the Addition of Equivalent Amounts of Iron and Protein, in the Form of Ferric Chloride, Casein and Liver, to the Basal Milk and Salmon Bread Diet

Periods of 1 week Diet per day in gm.	Weight	Plasma volume	R.B.C.	R.B.C. hematocrit	Hemoglobin: Level	Hemoglobin: Bled	Hemoglobin: Produced above basal rate	Food intake per kg. per day: Iron Total	Iron Added	Protein Total	Protein Added	Calories	Copper
	kg.	*cc.*	*million*	*per cent*	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*gm.*	*gm.*		*mg.*
Experiment F													
Bread S 215 Milk 430	15.3	–	7.3	26.2	58	2.0		0.43		2.2		75	0.06
" 250 " 430	15.7	926	6.6	30.2	55	1.2		0.49		2.3		82	0.07
" 250 " 430 Iron 0.03	15.4	–	7.3	33.3	61	1.9		2.4	1.9	2.4	0	84	0.07
" 250 Milk 430 Iron 0.03	15.6	–	7.5	34.8	67	0.8		2.4	1.9	2.3	0	83	0.07
" 250 Milk 430	15.6	–	6.0	28.0	55	23.5		0.5		2.3		83	0.07
" 250 " 430	15.8	908	5.0	25.7	50	0.8		0.49		2.3		82	0.07
							19						
Experiment E													
Bread S 250 Milk 430	15.1	837	5.5	23.2	46	16.9		0.51		2.4		86	0.07
" 250 " 430	15.1	–	4.9	21.7	44	1.6		0.51		2.4		83	0.07
" 200 " 430 Casein 70 Iron 0.03	15.6	–	5.3	24.3	49	1.5		2.42	2.0	5.9	3.8	74	0.07
Bread 250 Milk 430 Casein 70 Iron 0.03	15.9	821	5.3	28.6	58	0.7		2.42	2.0	5.7	3.7	80	0.07
Bread 250 Milk 430	15.9	–	5.7	26.5	50	16.3		0.48		2.3		81	0.07
" 250 " 430	15.6	–	5.2	26.9	50	0.7		0.49		2.3		83	0.07
" 250 " 430	15.7	870	4.6	23.7	49	10.8		0.50		2.3		83	0.07
							20						
Experiment G													
Bread S 250 Milk 430	15.8	917	5.75	30.8	60	1.7		0.48		2.3		82	0.06
" 200 " 430 Liver 250 Iron 0.021	16.7	–	7.65	32.9	78	1.7		2.37	2.0	5.3	3.4	85	0.44
Bread 200 Milk 430 Liver 300 Iron 0.021	16.9	–	6.05	31.9	72	31.7		2.49	2.1	5.9	4.0	88	0.51
Bread 250 Milk 430	16.8	–	5.55	31.1	66	18.4		0.46		2.2		77	0.06
" 250 " 430	16.5	–	5.55	31.8	65	1.4		0.47		2.2		78	0.06
" 250 " 430	16.6	862	6.25	33.2	67	3.8		0.46		2.2		78	0.06
" 250 " 430	16.5	785	5.3	27.6	56	15.4		0.47		2.2		78	0.06
" 250 " 430	16.1	898	6.25	31.7	63	1.6		0.48		2.3		80	0.06
" 250 " 430	16.9	871	5.9	30.5	60	1.8		0.45		2.2		77	0.06
							60						

differs from the other dogs in the use of inorganic iron with the liver diets (necessary to obtain an iron and protein intake equivalent to the casein-iron periods). Table I gives in detail the weekly observations and the diet for three representative experiments on Dog 4. In these three feeding periods of iron, casein-iron and liver, the iron addition is constant at about 2 mg. per kilo per day and the protein addition in the last two experiments is constant at about 3.7 gm. per kilo per day. The excess hemoglobin produced above the basal level is for the inorganic iron experiment 19 gm., for the inorganic iron and casein period 20 gm., and for the addition of liver to the basal ration 60 gm. It is obvious that casein does not contain the important factor present in liver, since its addition to the diet does not improve on the response to iron alone. These studies confirm the reports from Whipple's laboratory of the greater effectiveness of liver than of inorganic iron for hemoglobin production in this chronic hemorrhagic anemia of dogs. Furthermore, in liver and iron feeding periods representing the same quantitative iron intake, the liver is more effective. Hence, the difference between liver and inorganic iron cannot be due to a larger dose or a better availability of iron in the liver periods. It is well to point out that the responses recorded are submaximal to the substance in question. Experiments in feeding larger amounts of each of these materials have yielded more hemoglobin than is recorded for this series in Chart 1. Such an assay of necessity must employ a submaximal response.

Chart 1 illustrates the differences among dogs in their powers of hemoglobin production which Whipple often mentions. Although Dog 1 received less iron in all experiments than Dog 4, its hemoglobin responses are more marked in the inorganic iron periods. The constant quantitative nature of this hemoglobin response is shown by Dog 4 (solid blocks in the chart) with three iron, two casein-iron and two liver experiments. Such an animal has, therefore, a hemorrhagic type of anemia with the accuracy and sensitivity necessary for assay purposes comparable to the nutritional anemia type obtained in the young rat by an exclusive milk diet (16).

DISCUSSION

As a further confirmation of the rôle of protein in the form of casein in hemoglobin production in this hemorrhagic anemia, two dogs, raised in this laboratory, were studied in a similar manner to the other four except that Cowgill's (17) dog diet was used in the place of salmon bread. Since only two dogs have been observed in this experiment, these observations will be reported only briefly. This Cowgill ration has the following composition: casein (Lister) 37.2 per cent, sugar 34.3 per cent, lard 21.7 per cent, cod liver oil 2 per cent, agar-agar 2.4 per cent, salt mixture (McCollum-Simmonds No. 185, without ferric citrate, the same salt mixture used in the salmon bread) 2.4 per cent. The nutritional properties of this diet are 4.7 calories per gm., 30.2 per cent protein, 2 mg. per cent iron and 0.13 mg. per cent copper. Two yeast vitamin tablets (Harris) were added to the daily ration (represents 0.03 mg. of iron). This diet was fed in amounts allowing the 80 calories per kilo of body weight as specified by Cowgill. This is a high protein diet which allows about 5 gm. of casein per kilo daily as compared to the 2 gm. of fish and cereal protein afforded by the salmon bread diet. Protein deficiency is less likely, therefore, to be a factor when this diet is fed. After exhaustion of the hemoglobin reserves by bleeding, these dogs showed the same constant basal hemoglobin regeneration rate of 2 gm. a week as shown by the dogs eating the salmon bread ration. Mayerson and Laurens (18) reported that anemic dogs would neither maintain their hemoglobin level nor survive long on the Cowgill ration. It should be noted, however, that a different salt mixture was used in their diet. Some anemic dogs have refused to eat the Cowgill diet in this laboratory, but all of the six dogs mentioned in this report ate this ration well. They maintained their weight on this diet and showed, as mentioned, the same constant basal hemoglobin regeneration rate. The animals which had previously been stabilized on the salmon bread diet, showed an excess hemoglobin production of about 5 gm. a week for a 2 week feeding period with the Cowgill diet. Since its iron content is lower than in the salmon bread diet, this last observation again suggests a deficiency in the latter ration. The availability of the iron contained in the two diets does not explain this difference, since the bipyridyl reaction (3) shows about 50 per cent availability for the iron of both diets.

If protein of the amino acid content of casein were one of the important factors, animals whose anemia was stabilized on the Cowgill ration might be expected to show a response to the addition of inorganic iron approximating more closely their response to liver than in the case of dogs living on the salmon bread diet. Accordingly fresh frozen beef liver (275 gm. daily) was substituted during 2 weeks for the casein in the Cowgill ration which kept the daily protein intake constant and at the same time allowed an additional iron intake, arising from liver iron, of about 10 mg. daily. Excess hemoglobin production of about 100 gm. occurred. The same amount of inorganic iron (10 mg. per day) was added to the basal Cowgill ration for 2 weeks. No measurably increased hemoglobin regeneration was observed. Again the important factor contained in liver for hemoglobin regeneration does not appear to be contained in Lister casein. Casein also failed to facilitate hemoglobin regeneration after hemorrhage in McCay's experiments with dogs and rats (21).

The method of preparation (19) of Whipple's secondary anemia liver extract is such (the residue remaining after the pernicious anemia fraction is removed) that innumerable substances could be the important factor. The isolation of the active factor or factors contained in liver for hemoglobin regeneration in chronic hemorrhagic anemia could be accomplished by the use of additions to the basal ration of the many substances present in liver. Considering in Table I the much higher intake of copper in the liver period than in either the iron or the casein-iron periods, the simulation of the quantitative intake of copper simultaneously with that of iron in inorganic form to the intake present during a liver feeding period is the next obvious step. The addition of other types of protein, perhaps nucleoprotein, other minerals, hormones and vitamins (20) may perhaps be necessary to duplicate in this synthetic manner the hemoglobin response to liver feeding.

CONCLUSIONS

1. A group of dogs on a standard salmon bread diet with a slowly regenerating anemia were studied. The addition of liver to this diet during a 2 week period promoted a definitely greater regeneration of hemoglobin than did the addition of an amount of inorganic iron which was equivalent to that contained in the added liver. The more effective result attained with liver cannot, therefore, be attributed solely to the iron intake.

2. The greater response to liver is not due to its content of amino acids which are present in casein, since a diet containing an exactly

similar amount of calories, iron and protein nitrogen, made up of inorganic iron and casein does not cause a greater response than that obtained by the addition of that amount of inorganic iron alone to the standard basal diet.

3. Furthermore, the salmon bread diet does not produce a deficiency of the amino acids represented in casein, since dogs eating the high protein (casein) Cowgill dog ration show the same basal hemoglobin regeneration rate and a similar greater response to liver than to inorganic iron. The Cowgill ration, however, supplies some non-ferrous factor involved in hemoglobin regeneration which is not contained, to as great a degree at least, in the salmon bread.

4. Whipple's chronic hemorrhagic anemia of dogs serves as an accurate assay method for measuring the hemoglobin producing power of a substance. Quantitatively reproducible responses can be obtained.

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