THE RELATION OF CHROMATIN TO HEMOGLOBIN AND BILIRUBIN.

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PLATE 44.

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Recent studies of body pigment metabolism have given rise to two questions which await correlation. One is the origin of hemoglobin and the other that of the formation of bilirubin from other sources than hemoglobin. It is commonly accepted that blood pigment is the precursor of bilirubin, but Whipple (1) has suggested that hemoglobin and bilirubin may be formed independently of each other, probably in the liver, the former "by a progressive synthetic grouping of amino-acids, iron and other materials, which finally results in the finished product hemoglobin."

However there is evidence that hemoglobin appears in conjunction with the development of the erythrocyte and that it is derived from the chromatin of the hematopoietic cells. Schmidt (2), and more recently Sabin (3), have observed the presence of hemoglobin in the developing blood cells, although A. B. Macallum (4) first described its origin from the chromatin of the hematoblast in both amphibia and mammals.

Macallum devised methods of identifying cellular iron by micro-chemical methods and was able to distinguish the iron in hemoglobin from that in the chromatin of cells. His results showed that the first trace of hemoglobin in the earliest stages of an erythroblast was in the mitotic figure when the cell was dividing. These observations were based upon the fact that the iron in hemoglobin cannot be "unmasked" and specifically stained, while that of chromatin readily yields its iron in the presence of mineral acids.

Since all the cells of the body contain chromatin, it is necessary to account for the fact that in these hemoglobin is not produced. Anson

and Mirsky (5) and Keilin (6, 7) have shown that all animal and vegetable cells (except anerobic bacteria) contain a respiratory pigment, called cytochrome, or haem. The function of this pigment is identical with that of hemoglobin and the following diagram illustrates Keilin's conception of the relation of cytochrome and hemoglobin to cell respiration in the body:

R being the oxidized substance in the cells.

The cytochrome or haem of the ordinary cell is then the analogue of the hemoglobin in the erythrocyte. It is logical to suppose that cytochrome is derived from the chromatin of the cells containing it and that on the death of the cell it yields bilirubin in the same manner as hemoglobin. A diagram illustrating this conception of the relation between the respiratory and bile pigments might be constructed as follows:

It is evident from this discussion that the theory of a primary "pigment complex" as expressed by Whipple (1) need not be postulated because chromatin itself wherever its location is probably the precursor of both hemoglobin and the respiratory pigment of the cells, and from these bilirubin is derived.

The relation of chromatin to hemoglobin formation could be strengthened further if it were possible to demonstrate a quantitative relationship between the rate of regeneration of hemoglobin to the chromatin content of the bone marrow cells in an animal with anemia due to hemorrhage. This possibility has been examined in work now to be described.

It was shown in a previous paper (8) that hemoglobin formation could be inhibited in an anemic animal by iron starvation. McMaster and Haessler (9) were able to show that body iron depletion causes a depression of red blood cell formation in the bone marrow. It follows then that by modifying the amount of iron available to the body cells one should be able to stimulate or inhibit bone marrow cell growth as well as hemoglobin formation.

Methods.

The general plan of study followed the methods previously described by Whipple and Robscheit-Robbins (10), dogs being used as experimental animals. The bone marrow was studied first, during a period of rapid blood regeneration and second, during a period of relative iron starvation, while the peripheral hemoglobin level was held stationary. Blood regeneration was stimulated by feeding iron citrate or raw liver in relatively large amounts.

In the study of the relation of iron to hemoglobin formation in the bone marrow the methods described by Macallum were followed in making microchemical stains of the bone marrow cells. The marrow from the femur was chosen as representative because the decalcification of rib made the specimen unfit for the staining of iron. In each instance marrow from several parts of the bone were prepared and sections were cut also from the spleen and liver. The fresh tissue is fixed in 95 per cent alcohol, sectioned, and then placed in a solution of mineral acid and 95 per cent alcohol. Nitric, hydrochloric or sulphuric acid may be used, preferably the latter in 4 per cent concentration. After a short period (12 to 24 hours depending on the nature of the tissue) the sections are stained by applying a mixture of freshly made ammonium sulphide, and glycerine to the tissue and heating this preparation at a temperature of 55° for several days. The iron containing parts of the cell will then appear green in color while any substance containing iron not capable of being unmasked in the process will remain unstained. Of the several methods of demonstrating iron in tissue, it appeared after considerable experimentation that the ammonium sulphide-glycerine method was the most suitable. The sections were also stained with an aqueous solution of 0.5 per cent hematoxylin but the clearing of the section permitted by this method did not compensate for the lack of uniformity which they presented owing presumably to an inconstant hydrogen ion concentration (11). The steps in the preparation of tissue for examination were as follows:

1. Fixation in 95 per cent alcohol 24 hours.
2. Absolute alcohol 12 hours.
3. Xylol and paraffin.
4. Sectioning (6 M thick).
5. Place on slide without adhesive.
6. After drying dissolve paraffin in xylol and place in acid alcohol 24 hours with sealed cover.
7. Wash in 95 per cent alcohol thoroughly.
8. Place one drop of ammonium sulphide and one drop of glycerine on preparation, mix with glass rod and seal with cover slip.
9. Place preparation in warming oven 4 days.

Seven dogs were used in the experimental work but only the protocols are given of those from which photographs were taken. They were fed a bread mixture consisting of starch, bran, tomatoes, wheat flour, sugar and yeast. The iron content of the bread mixture was found to be 0.0055 per cent. The dogs were allowed only distilled water, and during the last 7 to 10 days of a period in which it was desired further to restrict the iron intake only milk was given the animals. These steps insured a rather high degree of iron restriction.

All the dogs had been continuously used in other experiments for a year prior

to sacrificing, and the hemoglobin had been maintained at a low level during this period. Dog 4 was fed 300 gm. of raw beef liver daily until hemoglobin production had reached a maximum. The dog was killed and the bone marrow removed by splitting the shaft of the femur and removing the bone marrow intact for hardening. In Dog 10 the hemoglobin level was receding without the necessity for hemorrhage at the time the animal was sacrificed, while in Dog 13 ferric citrate was used to increase hemoglobin production before studying the iron content of the bone marrow.

TABLE I.

Rate of Regeneration of Hemoglobin When Bone Marrow Was Studied.

Date	Red blood cell count	Oxygen capacity	Hb	Blood drawn	Hb	Hematocrit, per cent cells	Wt.	Diet
Dog 4								
1928	millions	cc.	per cent	cc.	gm.		kg.	
Feb. 20		10.4	56.3	20	1.5	29	12.9	Stock bread plus raw beef liver 300 gm. daily
" 23	6.54	11.1	60	20	1.5	30		
Mar. 1		10.6	57.3	100	7.9	30		
" 5		11.7	63.3	100	8.7	32	13.0	
" 7								
			Dog killed					
Dog 10								
Mar. 8		10.5	56.8	100	7.8	28	13.0	Whole milk only
" 15	5.52	9.5	51.4	15	—	24		
" 22		8.7	47.0	15	—	23		
" 22								
			Dog killed					
Dog 13								
Jan. 4	6.09	9.2	50	20		26		Bread plus Fe cit. 50 cc., 0.5 per cent
" 25		12.4	66	215	19.9	34		
Feb. 6								
			Dog killed					

The tissue blocks were cut at intervals during progress of the experiment, but staining procedures were carried out simultaneously on tissues on which comparisons of the staining reactions were to be made. The sections, usually four from each animal, were carried through the procedure to the warming oven at the same time, and when photographs were desired these were all made on the same morning for the entire set under identical conditions as to light, exposure, magnification, filters and development. Macallum found that the use of a steel blade in cutting

sections did not alter the staining depth although the handling of sections was carried out by means of glass rods always in glass containers. Study of individual cells was not undertaken since it was desired only to demonstrate the possible relation of rapid hemoglobin production to the amount of iron in bone marrow cells generally. It was necessary to observe in the sections that the green color due to iron was confined to the nuclei because any general diffusion of the color which occurred was evidence that the section was improperly prepared and must be discarded.

A direct estimation of the iron balance in dogs is impossible because of the large content of hair in the stools. However work in progress shows that a considerable depletion of the normal level of blood serum iron takes place under these conditions.

RESULTS.

The accompanying photomicrographs show the relative condition of the bone marrow with regard to its chromatin content. The sections demonstrated clearly that the iron content of the hematoblast is dependent on its chromatin content and that this in turn can be modified by iron feeding under the conditions of the experiments. In the animals in which the hemoglobin level is being barely maintained, nuclear chromatin stain is pale, while in those in which regeneration of hemoglobin was proceeding rapidly a heavy iron stain in the bone marrow is demonstrated.

The same phenomenon was present in sections from the spleen but in the liver the differences were not so manifest. As compared with the normal animal, however, all the tissues were poor in iron when anemia was present, and regardless of iron intake. Stains made of the liver and spleen of anemic dogs gave a much weaker chromatin reaction than those obtained from normal animals.

The interpretation of the depth of staining which different body cells exhibit after being subjected to the technique has been carefully considered. Since the chromatin is confined to the nuclei of the cells, the richness of its network is localized and the staining method itself prevents diffusion of iron into the surrounding tissues because water is not used in the preparation of the specimen. In order to further safeguard against mistakes in the subjective interpretation of the depth of staining only the two extremes in iron content were used. The bone marrow was either causing rapid production of hemoglobin or the hemoglobin level had remained stationary for a long period of

time under a constant stimulus. Hueck (12) has demonstrated a true comparison between the microchemical stain and chemical analysis of the tissues and his results have been confirmed in this laboratory by having a disinterested observer compare stained sections with the chemical analysis of tissues. The photomicrographs were made with the use of a red filter over a field large enough to include many cells.

The hypothesis that body cell chromatin, through cell metabolism, becomes the primary pigment complex is in no way antagonistic to the prevalent theories of pigment formation. It simply utilizes the facts already available and reduces the question to simpler and more basic considerations, whereupon investigation may continue.

SUMMARY.

1. Attention is directed to the diversity of opinion among investigators regarding the site and manner of hemoglobin formation in the body and its relation to bile pigment metabolism.

2. It is probable that in forming new hypotheses on this subject the earlier work of A. B. Macallum on the relation of chromatin to hemoglobin formation has not received sufficient consideration.

3. It has been shown by means of microchemical iron stains of the bone marrow cells, that the iron content of the hematoblast is increased during rapid hemoglobin production in simple anemia.

4. This fact is compatible with the work of Macallum who believed that hemoglobin is derived from the chromatin of the hematoblast. It does not support a theory that hemoglobin is formed as a part of a circulating pigment.

5. It is suggested that bilirubin is derived from the chromatin of body cells through the intermediary stages of the respiratory pigments, hemoglobin and cytochrome, from erythrocytes and other cells, respectively.

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EXPLANATION OF PLATE 44.

FIG. 1. Dog 13, fed Fe citrate. Bone marrow, femur showing increased density of cells. Preparations cannot be cleared for photographs. Red filter used. Oil immersion with No. 10 ocular. Sulphuric acid alcohol 24 hours. Ammonium sulphide-glycerine.

FIG. 2. Dog 10, iron starved. Same as above showing lessened density of bone marrow cells.

FIG. 3. Dog 4, fed liver. Showing moderate increase in chromatin network.

(Riecker: Relation of chromatin to hemoglobin and bilirubin.)