II. HEMOGLOBIN AND BILE PIGMENT OVERPRODUCTION IN THE SPLENECTOMIZED BILE FISTULA DOG

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In an earlier paper from this laboratory (4), it was pointed out that the splenectomized bile fistula dog showed cycles of bile pigment overproduction and anemia. Because of the very great amounts of bile pigments produced under these conditions we studied this reaction in considerable detail with the hope of a better understanding of body pigment metabolism. Our first paper (4) came to the conclusion that some obscure *intrinsic* factor related to the spleen was responsible for this reaction. The accompanying paper gives strong evidence that an *extrinsic* factor (*Bartonella canis*) is responsible. Splenectomy prepares the way for the *Bartonella* infection.

Granting that *Bartonella* infection explains the blood destruction with resulting surplus bile pigment production, the mechanism of hemoglobin production under these conditions is of peculiar interest to students of anemia. These dogs must produce enormous amounts of new hemoglobin and red cells during an active cycle on a diet which permits of but little new hemoglobin formation in simple anemic dogs. We have suggested that from the released hemoglobin the pyrrol aggregate is split off to form bile pigment while the globin fraction is turned over to form new hemoglobin. This proposal assumes that the body can readily produce a large excess of the pyrrol aggregate (four pyrrol rings) and there is much evidence (1) to support this argument.

To gain further information about blood destruction in bile fistula dogs we carried out a prolonged experiment with acetyl phenylhydrazine (Table 23) which gives similar results and supports the *Bartonella* observations.

Methods

The renal type of bile fistula was used as devised by Kapsinow, Engle, and Harvey (3). This type of fistula has been utilized for several years in this laboratory and the care of these dogs has been described in detail elsewhere (7). We emphasize the fact that such dogs with bile flowing freely into the renal pelvis can be maintained in perfect health and weight equilibrium for years. It is necessary to give 50–75 cc. of bile daily, together with a balanced ration.

The spleen may be removed at the time of the bile fistula operation or subsequently. Dogs are kept in metal metabolism cages at all times and water is given by stomach tube about 3 hours before the 24-hour urinary collection is made. The dog usually empties the bladder between the water ingestion and urine collection which makes for uniformity in urine collection, as obviously residual urine may give irregular values for daily bile pigment elimination. Catheterization would introduce infection and is never employed. Chloroform 5 cc. placed in the collection bottle acts as a preservative.

The dogs are weighed 3 times each week. Red cell hematocrit and blood hemoglobin determinations are made at least once a week and often daily during periods of pigment overproduction and anemia (5). Occasional blood plasma volume determinations are done by the vital red method (2).

Diets are essentially the same in each experiment and consist of canned salmon, Klim (a commercial dried whole milk powder), and a bread prepared in this laboratory. Water 400 cc. is added to the dried bread and the ingredients mixed into a mash. The bread is used in our anemia colony and is an adequate diet capable of maintaining dogs in health indefinitely. It contains wheat flour, bran, potato starch, canned salmon, sugar, cod liver oil, canned tomatoes, yeast, and a salt mixture. Its preparation has been described (10). On this diet the hemoglobin production of anemic healthy dogs has been carefully studied and is well understood. The obvious advantage of this diet in bile fistula dogs for bile pigment study needs no comment. An output of 2–5 gm. of hemoglobin each week over and above the maintenance factor is to be expected on this diet.

Bile Pigment Determination in Urine.—The urine-bile mixture passed in the 24-hour period is measured, and if from previous determinations the amount of bile pigments is known to be high the urine may be diluted with water to a definite volume.

Ten and 20 cc. portions of the diluted or undiluted 24-hour urine samples are made alkaline to litmus with ammonia, then 10 cc. of a calcium chloride solution (10 gm. in 100 cc. of solution) are added. This causes a voluminous precipitate to form, which is thrown down by use of the centrifuge, the supernatant clear fluid is decanted and the precipitate is dissolved in 10 cc. of 95 per cent alcohol to which 2 cc. of concentrated hydrochloric acid are added. The solution is poured into a 50 cc. volumetric flask, concentrated nitric acid containing oxides of nitrogen is added (0.2–0.4 cc.) and then made up to volume with alcohol. The blue-green color develops within a few minutes and the pigments are estimated by means of the colorimeter—Dubosque type.

One of the essential factors of the method is the presence of oxides of nitrogen in the nitric acid. This is of the greatest importance in order to insure proper development of the blue-green color. Pure nitric acid will produce the color reaction but it does so slowly. When the oxides of nitrogen are present in the nitric acid its oxidizing power is greater so that the bile pigments are oxidized to the blue-green phase within a few minutes. Amounts of this acid ranging from 0.2–0.4 cc. prove sufficient for the development of the end-point color and still do not cause the pigment to be oxidized rapidly beyond the blue-green phase. If, upon addition of the above amount of acid, the color development is slow, it may be hastened by immersing the flask in hot tap water with subsequent cooling to the correct volume.

Since the amount of bile pigment in the urine varies for different dogs it necessitates slight variations in the amount of acid needed. By adding the nitric acid 0.1 cc. at a time with a few minutes' interval one can observe the rapidity of color development and easily determine the amount of acid necessary.

The standard for bile pigment is made up 0.3 cc. of a solution of potassium bichromate (1 gm. in 100 cc. of water) plus 40 cc. of a solution of copper sulfate (10 gm. of $CuSO_4 \cdot 5\ H_2O$ to 100 of water). This standard solution was described by Rous and McMaster (6) and in this laboratory is standardized against samples of pure bilirubin prepared by Eastman Kodak Company. From this standard solution colorimetric readings enable the investigator readily to estimate milligrams of bile pigment in any solution. The milligram equivalents of bile pigments which this standard solution represents depend on the purity of the sample of bilirubin used in standardization.

A Wratten filter, No. 72, is employed in reading the colors, since urinary pigments themselves give a tint which without the filter make color matching difficult.

In the determination of bile pigments in whole bile, 1 cc. of bile is added to 49 cc. of the acid alcohol reagent in a volumetric flask, mixed, and allowed to stand at ice-box temperature until the blue-green color develops. The time necessary is about 20 hours, but would be shortened if flasks were kept at room temperature. The acid alcohol solution consists of 16 cc. concentrated hydrochloric acid, 2 cc. of nitric acid containing traces of the oxides of nitrogen made up to 1000 cc. with 95 per cent ethyl alcohol.

EXPERIMENTAL OBSERVATIONS

Bartonella Dogs.—Several animals of this type have been studied, and in so far as the blood picture and the pigment values are concerned, the results have been similar. We summarize here studies on one such animal extending over periods of 65 and 134 days. Such periods are characteristic of all animals observed.

Chart E shows the bile pigment and hemoglobin variations in a typical animal (31-359). This dog had been operated upon 80 days

previous to the beginning of the chart. The bile pigment and hemoglobin during the postoperative interval had remained within levels corresponding to the first 15 days shown on the chart. The abrupt rise of pigment output and the associated fall of hemoglobin are characteristic, as are the step-like swings of bile pigment to higher

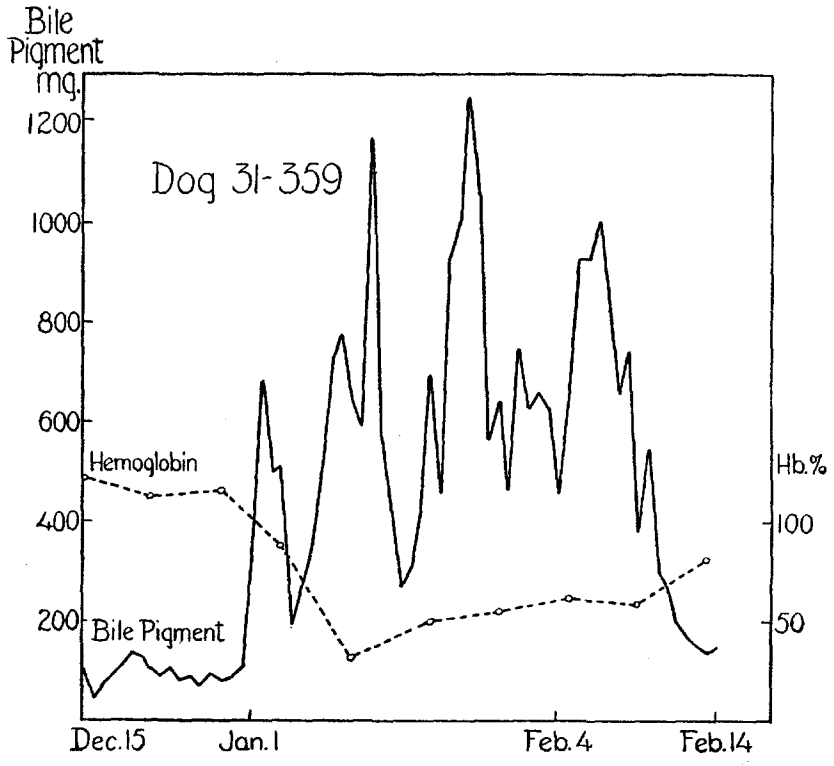

CHART E. Period of bile pigment overproduction and anemia in splenectomized bile fistula dog.

levels. During the period when the hemoglobin was very low, the dog was transfused several times in order to maintain life.

Table 21 is a summary of pigment data of the same dog for 134 days, during which time the animal was showing numerous cycles of blood destruction.

The various periods range, as shown in Column 1, from 6–25 days. They represent phases in which the pigment metabolism was relatively similar. In some of the periods a comparatively small amount of blood destruction was taking place; in others, large amounts of blood were being broken down. Column 2 shows the total amount of bile pigment estimated in the urine during the corresponding experimental period. The excess of pigment above the control level for a similar length of time is shown in Column 3. This control level is obtained by multiplying the number of days of the experiment at hand by the average daily output of bile

TABLE 21

Pigment Surplus in the Splenectomized Bile Fistula

Dog 31-359. Basal bile pigment output 87 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent	Pigment surplus gm. Hb. equivalent
	Total	Above control	Expressed as Hb.	Start	End	In urine	From blood	In blood	
	mg.	*mg.*	*gm.*	*per cent*	*per cent*				*gm.*
11	1,681	724	18	85	91	18		11	29
6	3,846	3,324	83	91	69	83	39		44
8	2,040	1,344	34	69	81	34		21	55
19	8,666	7,013	175	81	76	175	9		166
12	2,085	1,041	26	76	98	26		39	65
7	1,922	1,313	33	98	96	33	4		29
25	4,824	2,659	67	96	100	67		7	74
19	6,622	4,969	124	100	103	124		5	129
8	2,039	1,343	34	103	106	34		5	39
19	5,917	4,264	107	106	111	107		9	116
134	39,642	27,994	701			701	52	97	746
Per day.....	295	209	5.2			5.2	0.39	0.72	5.5

pigment during the control period, which in this case was of 9 weeks' duration. Average basal bile pigment output during this period was 87 mg. daily. The excess amounts of bile pigment expressed as hemoglobin, represented in Column 4, are obtained by dividing the figures in Column 3 by 40, 1 gm. of hemoglobin yielding 40 mg. of bile pigment. Column 7 shows identical figures with Column 4. All subsequent calculations are in terms of grams of hemoglobin. The pigment lost or gained in the blood is estimated from the fall or rise in hemoglobin, which is recorded in Columns 5 and 6. The grams of hemoglobin equivalent to the change in hemoglobin percentage are estimated by the formula:

Grams Hemoglobin = 13.8 × Blood Volume × Per Cent Hemoglobin Change,

100 per cent hemoglobin representing 13.8 gm. per 100 cc. of whole blood. Such differences are shown in Columns 8 and 9, as the pigment lost or gained in the blood.

The pigment surplus is represented in Column 10. This is estimated by the addition or subtraction, as the case may be, of the pigment change in the blood, to or from the excess of bile pigment in the urine. As is seen, the amounts of bile pigment at various periods bear no direct relation to the change in the hemoglobin level during the same periods. In addition, there is no constancy between the figures representing the pigment surplus and either the bile pigment or the hemoglobin values.

In summarizing this table, it is seen that the total time of the experiment was 134 days, during which time the dog eliminated 39,642 mg. of bile pigment. This amount is 27,994 mg. above the normal control level for the animal, and represents an equivalent of 701 gm. of destroyed hemoglobin. During this interval, 97 gm. of hemoglobin were built up, and 52 gm. of hemoglobin were destroyed, as measured in the blood stream. Thus the dog had, at the end of the period, 45 gm. more circulating hemoglobin than at the start of the experiment. This, plus the hemoglobin equivalent for the bile pigment which was eliminated, gives a total pigment excess of 746 gm. This represents a daily average formation of 5.5 gm. of hemoglobin.

Phenylhydrazine Experiments.—Dog 31-267 with a gall bladder-renal fistula was used in these experiments. It was given acetyl phenylhydrazine hydrochloride subcutaneously, in an attempt to determine whether the destruction of blood by this drug would produce pigment changes in any way similar to those of the *Bartonella* animals. This animal was kept under experimental conditions similar to the other dogs.

Table 22 represents a fore period in the course of the experiment, which is followed by an interval in which three doses of 150 mg. each of the drug were given. In this fore period 123 mg. of bile pigment were eliminated daily on an average as compared with the basal control level of 66 mg. This higher pigment level is due to the fact that this period followed a previous injection of phenylhydrazine and the control level had not been attained before more drug was injected. As is shown, the daily bile pigment elimination increases, while the blood hemoglobin percentage falls immediately following the injections. The excess bile pigment above the fore level averages 155

mg. a day which is equivalent to 4 gm. of hemoglobin. During the experimental period there was a drop in hemoglobin from 121 per cent to 80 per cent, which is equivalent to a loss of 44 gm. of circulating

TABLE 22

Acetyl Phenylhydrazine Blood Destruction and Bile Pigment Output

Dog 31-267.

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Weight	Amount hydrazine injected
1932	*mg.*	*per cent*	*cc.*	*kg.*	*mg.*
Oct. 16	125		510		
17	138		430	13	
18	105		660		
19	127		495	13.2	
20	127	121	490		
21	158		640	13.2	
22	112		720		
23	96		600		140
24	272		330	13.3	
25	191		720		
26	160		400	13.2	
27	229	104	510		
28	167		890	13.2	150
29	259		450		
30	359		500		
31	309		610	13.1	
Nov. 1	261		540		
2	283	87	500	13.1	
3	191		590		
4	392		450	13.1	150
5	304		570		
6	370		490		
7	467		450	13.2	
8	263		750		
9	256	80	470	13.2	

Diet = 250 gm. salmon bread—food consumption 100 per cent.
75 gm. salmon.
30 gm. Klim.

hemoglobin. (The blood volume of the animal was 952 cc.) 44 gm. of destroyed hemoglobin, theoretically, would cause 1760 mg. of bile pigment above the fore level to be eliminated. The actual recovery

was 2790 mg. or 1030 mg. in excess of the expected amount. Such excess bile pigment elimination is quite similar to that found in the *Bartonella* dogs.

Table 23 is constructed similarly to Table 21, and illustrates the results of continued injections of acetyl phenylhydrazine over comparatively long periods of time. The average dose of the drug was 100 mg. injected subcutaneously every day or two. In the first part

TABLE 23

Pigment Surplus with Hydrazine Blood Destruction in Bile Fistula

Dog 31-267. Basal bile pigment output 66 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent	Pigment surplus gm. Hb. equivalent	Hydrazine amount injected
	Total	Above control	Expressed as Hb.	Start	End	In urine	From blood	In blood		
	mg.	*mg.*	*gm.*	*per cent*	*per cent*				*gm.*	*gm.*
36	5,464	3,088	77	128	94	77	44		33	0.24
21	1,383	−3		94	129			45	45	0
23	5,985	4,467	110	104	85	110	39		72	0.60
7	1,400	938	23	85	80	23	7		16	0.20
8	2,109	1,581	40	80	94	40		19	59	0
21	6,817	5,431	135	94	53	135	54		81	0.78
17	1,757	635	16	53	86	16		43	59	0
31	7,520	5,474	137	82	46	137	47		90	1.5
21	5,676	4,290	107	46	57	107		14	121	1.0
35	11,784	9,474	237	57	59	237			237	2.8
21	2,723	1,337	33	59	106	33		62	95	0
241	52,618	36,712	915			915	191	183	908	
Per day.....	218	152	3.8			3.8	0.79	0.76	3.7	

of the experiment a few larger doses of 200–300 mg. were injected but they caused too rapid hemolysis of blood and the dog became upset and consumed only portions of its food.

Comparison of the two tables shows that the pigmentary changes are similar. Again there is no direct relation between the amounts of bile pigment and changes in the hemoglobin level. During the experimental period of 241 days the dog eliminated 36,712 mg. of bile

pigment in excess which is equivalent to 915 gm. of hemoglobin. During this interval 191 gm. of hemoglobin were destroyed and 183 gm. of new hemoglobin were formed as measured in the blood stream. The total excess of pigment formed amounts to 908 gm. expressed as hemoglobin equivalent or a daily average formation of 3.7 gm. of hemoglobin.

In those periods when no drug was injected the bile pigment elimination decreased and there was a rapid increase in the amount of circulating hemoglobin showing how readily the dog could rebuild hemoglobin from the conserved products of destroyed red cells. Undoubtedly new hemoglobin was being formed during the other periods but this fact cannot be brought out from simply measuring the amount of circulating hemoglobin.

DISCUSSION

Hemoglobin production holds the spotlight in this paper and interesting possibilities suggest themselves to explain the observed facts. During active cycles of red cell destruction (*Bartonella*) these bile fistula splenectomized dogs will produce over 5 gm. of new hemoglobin per day or a total of 140 gm. per 2 weeks. In simple anemia dogs this large hemoglobin output (9) can be attained only by a very favorable diet intake (liver plus iron). On the standard basal ration (salmon bread) in simple anemia dogs we expect 5–10 gm. hemoglobin output per 2 weeks. The splenectomized bile fistula dogs are fed this basal ration.

At present the most satisfactory explanation is that the hemoglobin released from destroyed cells is promptly split with escape of the pyrrol aggregate (four pyrrol rings) as bile pigment and the globin fraction is recaptured or turned over to form promptly the new hemoglobin for the red cells in the marrow. The iron presumably goes along alone or with the globin to the make-up of this new hemoglobin.

If one is not wholly satisfied with the preceding explanation it may be argued that not all of the bile pigment comes from hemoglobin but that some of the pyrrol aggregate formed within the body is turned out as excess bile pigment. In any case it must be admitted that the body can supply a large surplus of pyrrol aggregate whether it is all built up into new hemoglobin or some of it is shunted directly into

bile pigment. There are some experimental data which support this last proposal.

Destruction of blood by phenylhydrazine *in vivo* gives a picture much like *Bartonella* blood destruction. It is of similar character but the new hemoglobin output is distinctly less in the hydrazine experiment cited above (Table 23). However we have reported (4) *Bartonella* experiments of only moderate severity which show an identical picture. More severe blood destruction could be induced by hydrazine but the dogs would be sick and refuse food which introduces a confusing factor. The hydrazine experiments support the argument that all or the great bulk of the bile pigment in these experiments comes from hemoglobin.

It is obvious that under the conditions of these experiments, the amount of hemoglobin turnover in the body at any one time cannot be estimated by studies of the blood alone. The ordinary methods for blood hemoglobin determination show the total increase or decrease of this substance in a given time. In the absence of more than normal rates of blood destruction *in vivo*, or in removal of blood from the circulation, such methods offer a satisfactory basis for estimating hemoglobin regeneration. In the presence of excessive blood destruction, however, no accurate idea of the actual amount of pigment turnover can be secured by these methods alone. Under such conditions, hemoglobin may be built up so rapidly, that although comparatively large amounts are destroyed, the blood hemoglobin level in a given interval may show only a slight drop or even an actual rise. In such cases, the estimation of the amount of bile pigment eliminated is of great value.

Other observed facts which are related to this debate should be mentioned. In this laboratory it has been found that in standard anemic dogs hemoglobin introduced intravenously will be quantitatively returned as new hemoglobin in new red cells. This reaction follows whether we use dog or sheep or goose hemoglobin in anemic dogs (8). In the normal healthy bile fistula dog hemoglobin introduced intravenously results usually in quantitative appearance (7) of bile pigment in the proportion of 1 gm. hemoglobin = 40 mg. bile pigment. But in the *anemic* bile fistula dog (1) hemoglobin given intravenously results in an apparent paradox. There is a quantitative recovery of the hemoglobin which appears as new hemoglobin

in the new red cells just as in the standard anemic (non-fistula) dog. There is also almost a quantitative escape of bile pigment just as in the non-anemic bile fistula. There must be a surplus production of the pyrrol aggregate whether this is used in the manufacture of new hemoglobin or of bile pigment or both. This experiment therefore supports the *Bartonella* and hydrazine experiments tabulated above.

The *pyrrol aggregate* (4 pyrrol rings) is the essential nucleus of the pigment radicle (hemin) of hemoglobin as well as of bile pigment and related body pigments. The body can produce considerable excess amounts of this pyrrol aggregate on demand. From what source is it derived? From reserve stores? In long continued anemia it would seem that all pigment stores would have been exhausted when anemia of severe grade (⅓ normal hemoglobin) is continued for 3 to 5 to 7 years. From diet? But only in certain peculiar conditions does this surplus appear as the need arises and the diet is strictly uniform at all times. From body synthesis? We favor this probability as the reaction occurs so promptly with the formation of such large amounts when the need is present. The required amino acids presumably would come from the diet but we cannot believe that this intact pigment nucleus can be concealed as such in the diet under consideration.

Globin makes up approximately 95 per cent of the hemoglobin molecule and we believe its importance is in proportion to this figure. The great bulk of the studies dealing with hemoglobin consider mainly the pigment radicle. The observations above indicate that the pigment radicle can be produced readily in abundance. At times iron may be a determining factor. Probably the globin fraction determines the capacity of the body to produce new hemoglobin under many conditions. How and where is globin produced in the body and what materials go into the synthesis of this large molecule? The source and internal metabolism of globin is shrouded in mystery. Any observed facts related to globin within or without the body will be significant and in time should lead to a better understanding of hemoglobin metabolism.

SUMMARY

Blood destruction associated with *Bartonella* or with a drug (hydrazine) in bile fistula dogs yields a large pigment excess. These dogs form large amounts of new hemoglobin and bile pigment on a diet

which permits of but little new hemoglobin production in standard anemic dogs.

When hemoglobin formation and hemoglobin destruction are occurring rapidly and simultaneously, estimations of the percentage of circulating hemoglobin alone, though showing the eventual *total* increase or decrease of this substance, do not permit one to determine the actual amounts formed or destroyed.

It is suggested that the body can produce readily a large amount of the pyrrol aggregate (four pyrrol rings) which may go to form new hemoglobin. At the same time the globin is probably saved from destroyed red cells and turned over into new hemoglobin for new red cells.

It is certain that globin may be a determining factor under certain circumstances in the construction of new hemoglobin for new red cells. Our knowledge about the construction and internal metabolism of globin is extraordinarily limited.

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