BILE AND BLOOD PLASMA CHOLESTEROL AS INFLUENCED BY BLOOD DESTRUCTION IN NORMAL AND BILE FISTULA DOGS

By A. WRIGHT, M.D., and W. B. HAWKINS, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

(Received for publication, February 14, 1938)

It is commonly assumed that part of the blood plasma and bile cholesterol has its origin from destroyed red cells. Search of the literature reveals no convincing data that warrant such an assumption.

Under standard conditions the normal bile fistula dog eliminates bile pigments ranging in amount from 60 to 120 mg. a day. The individual dog maintains a remarkably constant daily output. This bile pigment has two possible known sources, namely from the hemoglobin of red cells or muscle. Whipple and Robscheit-Robbins have demonstrated that dog muscle hemoglobin when given parenterally may be converted into bile pigment (12). How much bile pigment is derived from the daily disintegration of myohematin is not known. However from evidence accumulated from other studies relating to the length of the life of circulating red cells it would appear that the greater part of the bile pigment daily excreted comes from the wear and tear disintegration of the red blood cells (5).

Theoretically 1 gm. of hemoglobin is equivalent to 40 mg. of bile pigment and it has been shown that following the intravenous injection of dog hemoglobin solutions there is an increase in bile pigment elimination corresponding to 90 to 95 per cent of the expected increase if there were a quantitative transformation of the pigment radicle of hemoglobin to bile pigment (10).

Brun (2) has determined that red cells contain 140 mg. per cent of cholesterol, all of which is in the uncombined form. Since normally our dogs have a circulating hemoglobin of 130 per cent or 18 gm. (standard is 13.8 gm. per 100 cc.) then 5 cc. of blood would contain

approximately 1 gm. of hemoglobin and 4 mg. of cholesterol in the contained red cells.

If we disregard myohematin as a source of bile pigment and assume that all of this pigment comes from destroyed red cells, then at most only from 1.5 to 3 gm. of hemoglobin are being destroyed daily on the basis of 60 to 120 mg. of excreted bile pigment. This amount of hemoglobin represents from 8 to 15 cc. of red cells and with their destruction 6 to 12 mg. of cholesterol would be liberated. This amount represents a very small fraction of the total plasma cholesterol which ranges between 100 to 200 mg. per cent.

During the course of several experiments utilizing normal and bile fistula dogs, large amounts of red cells have been destroyed by means of acetyl phenylhydrazine or due to infection of the dog by *Bartonella canis* (7). Daily determinations of plasma cholesterol, bile cholesterol, and bile pigments have been made. These data indicate that *following the destruction of large numbers of red cells there is no corresponding increase in the plasma cholesterol.* There may be actually a decrease in the bile cholesterol at the height of blood destruction when much excess bile pigment is being excreted.

Methods

Two types of bile fistula dogs have been used, the sterile bag fistula as devised by Rous and McMaster (9) and the gall-bladder renal fistula of Kapsinow, Engle, and Harvey (6). In the latter fistula the common bile duct is ligated and cut and the fundus of the gall-bladder is sutured into the renal pelvis. The dogs are kept in galvanized iron metabolism cages and the urine-bile mixture is collected over 24 hour periods. Chloroform 5 cc. is used as preservative. Such fistula dogs can be maintained in excellent physical condition for long periods of time if they are fed properly and if dog or ox bile (50 to 75 cc.) is given daily. The bile is given either by stomach tube or mixed with the food and it is beneficial in preventing abnormalities resulting from total bile deprivation (4).

The dogs are fed either kennel diet or salmon bread mixture. The former consists of mixed hospital kitchen scraps, and the salmon bread, baked in the laboratory, contains wheat flour, potato starch, salmon, tomatoes, bran, and a salt mixture. Its preparation has been previously described (11). These diets are suitable for bile fistula dogs as they are rich in carbohydrates, adequate in protein, but low in fat.

Methods of hemoglobin, blood volume, and bile pigment determination have been described elsewhere (11, 7).

The dogs are bled every morning at the same time and fed in the early afternoon

so the blood samples are free from the questionable influence of alimentary absorption. Approximately 10 cc. of blood drawn from the jugular vein are received into a 15 cc. calibrated hematocrit tube containing 2 cc. of a solution of 1.4 per cent sodium oxalate and immediately centrifugalized for 35 minutes.

Total plasma cholesterol has been determined following the method of Bloor (1) with slight modifications. The method for determining the cholesterol esters is given in detail since changes have been made in the method formerly used. The addition of ethyl ether as an extractive along with petroleum ether insures uniform and complete extraction of the cholesterol esters, whereas when petroleum ether alone is used, the values obtained are lower and tend to vary. The ethyl ether should be fresh and of purest anesthesia grade.

Method for the Colorimetric Determination of Blood Plasma Cholesterol.—Pipette 5 cc. of plasma into 75 cc. of alcohol-ether extraction mixture (one part ethyl ether to 3 parts of 95 per cent alcohol) in a 100 cc. volumetric flask. The plasma is run slowly into the extraction mixture as the flask is actively shaken; a fine white curd-like precipitate forms. The mixture is brought carefully to the boiling point (steam bath) for 30 seconds, stoppered, cooled to room temperature, and the volume made up to 100 cc. with the alcohol-ether mixture. The mixture is then filtered; the filtrate is placed in a tightly stoppered flask. The precipitate is discarded.

Determination of Total Cholesterol.—An aliquot[1] of the alcohol-ether extract is placed in a 125 cc. flask, a small stirring rod inserted, and evaporated to dryness on the steam bath. To the dry residue in the flask, 5 cc. of $CHCl_3$ are added, boiled down to about 2 cc. volume, and poured into a 10 cc. glass stoppered, graduated cylinder. This is repeated twice more. The final volume of the combined $CHCl_3$ extracts should not exceed 7 cc.

Color Development.—To the $CHCl_3$ extract in the graduated cylinder, add 2 cc. of acetic anhydride and 0.1 cc. concentrate H_2SO_4; stopper, mix gently, and make up to 10 cc. with $CHCl_3$. The color development is made at room temperature which should not vary appreciably from 25°C. The colorimetric readings are made 20 minutes after addition of the H_2SO_4. The color is maintained at its maximum for about 15 minutes, during which time colorimetry should be completed.

Determination of Esterified Cholesterol.—To an aliquot[1] of the extract in a 125 cc. flask, add 2 cc. of 0.5 per cent digitonin in 95 per cent alcohol, insert a glass stirring rod, and evaporate to dryness. To the residue in the flask add 25 cc. of petroleum ether (redistilled fraction boiling below 60°C.), boil this down to about 10 to 12 cc., and pour through a sintered glass filter under gentle suction into a

---

[1] *Aliquots.*—The amount of extract taken for an aliquot should closely approximate in cholesterol content the amount of cholesterol used in the standard. For most normal plasmas an aliquot of 10 cc. of extract for the total cholesterol and 15 cc. for esterified cholesterol is satisfactory when 5 cc. of plasma are used.

125 cc. flask. This is repeated 3 times more, using 25 cc. of ethyl ether instead of petroleum ether. (The sintered glass filter is of the type marked 4 G 4, Schott and Gen, Jena). The combined volume of the one petroleum ether and the 3 ethyl ether extracts is evaporated to dryness on the steam bath. The residue in the flask is extracted with $ChCl_3$ and the color developed in the method for total cholesterol.

Determination of Bile Cholesterol.—The method of Elman and Taussig (3) was used for the determination of cholesterol in bile with development of the color reaction as detailed in the method of blood plasma cholesterol.

EXPERIMENTAL OBSERVATIONS

Table 1 (dog 35-17) illustrates the results of a period of blood destruction with acetyl phenylhydrazine in a normal dog. This dog, an adult female mongrel, weight 16.8 kg., was fed the basal salmon bread diet beginning 10 days before control observations were made. After 7 days of observation for a control period, the dog was given subcutaneously on 2 consecutive days, 100 mg. of acetyl phenylhydrazine dissolved in normal saline. After this the red cell percentage began to decrease, there was marked bilirubinemia, and the urine was deeply pigmented. 3 days later the same amount of phenylhydrazine was given and then on alternate days for five doses. Through this period the hemoglobin decreased rapidly from a level of 136 per cent to 58 per cent. A slight increase in the blood plasma cholesterol was noted during the first 5 days of blood destruction. After the injections of drug were stopped, the bilirubinemia cleared rapidly and the hemoglobin increased to 103 per cent in 9 days. During the recovery period from the acute anemia there were no significant changes in the plasma cholesterol values. This experiment was repeated twice on each of two other normal dogs with similar results. Each one of these dogs lost during periods of acute blood destruction about one-half of its circulating red cells without appreciable change in the blood plasma cholesterol values.

Table 2 (dog 34-212) illustrates the results of a similar period of blood destruction in a bag fistula dog. The dog, an adult female hound, weight 17 kg., was fed a diet of kitchen scraps to which was added daily 100 cc. of ox bile. The dog maintained its weight and consumed all of its food through the experiment. After a 7 day control period, the dog was given acetyl phenylhydrazine subcutaneously

in 6 doses of 100 mg. each as indicated in the table. The excretion of bile pigment increased rapidly with continued administration of the drug, and the hemoglobin decreased from a level of 133 per cent to 59 per cent. During this period the blood plasma cholesterol values

TABLE 1

Blood Plasma Cholesterol in Normal Dog (35-17)
Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenyl-hydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*per cent*
Jan. 4		108	70	65	136
5		118	80	68	
6		111	73	66	
7	100	109	67	61	
9	100	123	86	70	135
10		161	109	68	
11		118	81	69	
12		121	87	72	
13	100	145	98	68	
14		119	82	69	
15	100	113	78	69	
16		117	91	78	85
17	100	104	69	66	
18		100	77	77	
19	100	108	72	67	
20	100	110	78	71	59
21		108	73	68	58
22		99	63	64	
23		104	75	72	
24		100	69	69	
25		105	71	68	
26		104	67	64	
27		121	79	65	
28		124	75	60	
29		122	74	61	
30		117	78	67	103

were slightly lower, but the percentage of esterified cholesterol remained unchanged. The bile cholesterol decreased from a level of around 30 mg. to a low point of 21 mg. on the days when the bile pigment elimination was high. Through the following period in which the hemoglobin returned to a level of 124 per cent the blood plasma

and bile cholesterol returned to the control levels. During the experiment 5360 mg. of bile pigment were excreted in excess over control amounts and this is equivalent to 134 gm. of hemoglobin destroyed (40 mg. bile pigment = 1 gm. hemoglobin). The quantity of blood

TABLE 2

Bile and Blood Plasma Cholesterol in Closed Fistula Dog (34-212) Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenyl-hydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Bile cholesterol	Bile pigments	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*mg.*	*mg.*	*per cent*
Oct. 26		201	157	78	30	129	133
27		221	167	76	33	73	
28		190	149	78	35	100	
29	100	201	160	79	32	73	
30	100	193	151	78	30	97	
31	100	210	152	72	31	131	
Nov. 1	100	197	135	69	32	250	102
2		182	142	78	27	238	
3	100	184	144	78	30	502	
4	100	176	146	73	30	726	
5		207	148	71	24	1020	
6		180	142	79	22	1102	59
7		182	140	76	21	857	
8		185	138	75	24	519	
9		181	133	74	25	319	
10		193	135	70	29	333	
11		189	135	72	30	304	
13		214	174	81	35	235	
14		212	166	78	39	161	
15		197	128	65	37	144	
16		195	145	74	34	89	
17		210	164	78	35	74	
18		211	155	74	33	95	124

represented by this amount of hemoglobin would contain approximately 536 mg. of cholesterol.

A similar experiment was repeated once again on this same dog and once on another bag fistula dog with comparable results.

Table 3 (dog 34-211) gives the data obtained from a renal fistula dog that weighed 18.5 kg. and was being fed the salmon bread diet plus ox

bile 50 cc. and dog bile 50 cc. daily. Food consumption was 100 per cent and the weight was maintained. After a 7 day control period the dog was given acetyl phenylhydrazine subcutaneously in ten doses of 100 mg. each over a period of 14 days. The bile pigment excreted increased rapidly and with continued administration of the drug the

TABLE 3

Blood Plasma Cholesterol in Renal Fistula Dog (34-211)
Blood Destruction by Acetyl Phenylhydrazine

Date	Acetyl phenyl-hydrazine	Total blood plasma cholesterol	Esters blood plasma cholesterol	Esters of total	Bile pigments	Hemoglobin
	mg.	*mg. per cent*	*mg. per cent*	*per cent*	*mg.*	*per cent*
Dec. 13		105	74	70	59	127
14		111	86	77	81	
16	100	102	67	66	66	
17	100	112	76	68	203	118
18		107	71	66	149	
19	100	96	63	66	320	
20	100	92	57	62	317	
21	100	94	60	64	338	
24	100	88	55	63	790	
27	100	83	51	62	742	60
28	100	84	59	70	637	
29	100	79	55	70	399	
30	100	71	43	61	427	
31		73	45	62	360	71
Jan. 2		83	60	72	288	
3		82	59	72	194	
5		87	62	71	172	
6		88	68	77	151	
7		91	66	73	227	99
8		83	57	69	144	
9		89	63	71	212	
10		99	73	74	120	106

hemoglobin decreased from 118 to 60 per cent and the blood plasma cholesterol levels showed a slight but definite decrease. During the following 12 days the total blood plasma cholesterol and esterified cholesterol values remained slightly lower than the control levels. The bile pigments returned to the control levels 2 weeks after the last day given in the table. This delay was due to the marked bilirubinemia resulting from the blood destruction. The bile pigment excreted

above control amounts during the experimental period of 39 days was 8829 mg. which is equivalent to 220 gm. of destroyed hemoglobin. With the destruction of this much hemoglobin approximately 880 mg. of cholesterol would have been liberated from disintegrated red cells.

This experiment was repeated twice on another gall-bladder renal fistula dog with similar results.

TABLE 4

Bile and Blood Plasma Cholesterol in Closed Fistula Dog (33-51) Blood Destruction by Bartonella canis

Date	Total blood plasma cholesterol	Bile cholesterol	Bile pigments	Hemoglobin
	mg. per cent	*mg.*	*mg.*	*per cent*
Nov. 17	158	29	103	106
18	127	36	84	
19	128	28	73	
20	145	14	85	
21	120	11	370	
22	105	9	815	
23	95	13	600	94
24	106	15	377	
25	93	16	474	
26	109	12	444	
27	94	16	392	
28	84	16	642	79
29	80	15	660	
30	87	13	727	
Dec. 1	83	13	724	
2	87	20	342	
3	137	19	174	
4	112	23	137	
5	132	31	116	100
6	120	18	127	
7	155	20	183	

Table 4 (dog 33-51) illustrates the result of blood destruction as the result of *Bartonella canis* infection in a splenectomized dog (7) carrying a bag fistula. It was a mongrel female weighing 17.5 kg. The diet was the salmon bread mixture and it was consumed and the weight was maintained throughout the experiment. 9 days after the operation at which the fistula was established and splenectomy performed,

the dog was given whole blood (5 cc.) intravenously from another splenectomized bile fistula dog with a demonstrated *Bartonella canis* infection. As indicated in the table the bile pigment rose markedly on the 5th day after the introduction of the infected blood.

In the following period large amounts of bile pigment were excreted and this was accompanied by bilirubinemia and a decrease in the circulating hemoglobin. *Bartonella canis* bodies were demonstrated in the red cells at this time. The excess of bile pigment eliminated amounted to 5927 mg. and this is equivalent to 148 gm. of hemoglobin. As the result of destruction of red cells containing the hemoglobin one might expect 592 mg. of cholesterol to be liberated. The blood plasma cholesterol during this period was slightly lower than the control level and the bile cholesterol much lower. As the bile pigments rose to a maximum the biliary cholesterol decreased markedly; as the pigment excretion decreased, the bile cholesterol increased until both constituents reached normal levels. This inverse relationship between the bile pigments and cholesterol was noted repeatedly in this dog during later periods of blood destruction and in another splenectomized bile fistula dog also infected with *Bartonella canis*. Although a considerable amount of blood is destroyed as the result of the infection, no increase in the blood cholesterol occurred during any of the periods.

DISCUSSION

As yet there is no conclusive evidence that indicates that bile pigment is formed except from the hemoglobin of muscle and red cells that is daily undergoing disintegration. Since these dogs were kept under standard conditions throughout the experiments, any excess bile pigment eliminated above the control level may be attributed to the destruction of red cells by the acetyl phenylhydrazine or due to the *Bartonella canis*.

In these normal and bile fistula dogs large numbers of red cells have been destroyed with consequent liberation of hemoglobin in a short period of time. Basing the amount of hemoglobin destroyed on the excess bile pigment eliminated, we may state that from 134 to 220 gm. hemoglobin have been broken down in periods of from 8 to 12 days. Since 1 gm. of hemoglobin is approximately equivalent to 4 mg. of cholesterol, then from 536 to 880 mg. of cholesterol should have been

liberated during the period of blood destruction. This amount even though spread over several days should be sufficient to raise the plasma cholesterol levels. If we take 200 mg. per cent as the higher level of cholesterol normally present in the plasma, then dogs of an average weight of 17 kg. and 800 cc. plasma volume would have not more than 1600 mg. of cholesterol in the circulating plasma. However the data show that there was no increase but rather a slight decrease during the periods of red cell destruction and anemia. The percentage of esterified cholesterol remains constant. With the development of marked anemia there is some dilution of plasma and the slight decrease of total cholesterol may be the result of this dilution and therefore have no true significance.

If under the conditions of marked and quite rapid red cell destruction there is no increase in circulating plasma cholesterol, certainly the assumption that the normal daily destruction of red cells is a factor in contributing cholesterol to the plasma is not warranted.

There is no question but what cholesterol is liberated as the result of red cell destruction but it is possible that the stroma and cholesterol of the destroyed cells are taken into the phagocytic cells of the body and there gradually metabolized for the benefit of red cell production, and consequently the cholesterol is not set free in the plasma.

It is interesting to speculate as to the cause of the decreased bile cholesterol during the periods of marked pigment elimination as noted repeatedly in the dogs infected with *Bartonella canis*.

This decrease is apparent also in the bile of the dogs made anemic by acetyl phenylhydrazine. During the period of marked jaundice, the liver cells may be mildly injured and consequently the function be deranged or it is possible that the bile canaliculi may be obstructed by bile pigments which are being eliminated in such excess amounts. Autopsies on other bile fistula dogs infected with *Bartonella canis* have revealed that many of the bile canaliculi are filled with plugs of bile pigment (8).

CONCLUSIONS

Destruction of large amounts of red cells in normal and bile fistula dogs by means of acetyl phenylhydrazine causes no significant alterations of the blood plasma but there is some decrease in the bile cholesterol.

During *Bartonella canis* infection the splenectomized bile fistula dog periodically breaks down large quantities of red cells with slight decrease in the plasma cholesterol and marked decrease in the bile cholesterol.

In the periods of blood regeneration following such acute anemias there are no significant alterations in the values for plasma or bile cholesterol.

BIBLIOGRAPHY

1. Bloor, W. R., *J. Biol. Chem.*, 1916, **24,** 227.
2. Brun, G., *Biochem. Z.*, Berlin, 1936, **287,** 420.
3. Elman, R., and Taussig, J. B., *J. Lab. and Clin. Med.*, 1932, **17,** 274.
4. Hawkins, W. B., and Whipple, G. H., *J. Exp. Med.*, 1935, **62,** 599.
5. Hawkins, W. B., and Whipple, G. H., *Am. J. Physiol.*, 1938, **122,** 418.
6. Kapsinow, R., Engle, L. P., and Harvey, S. C., *Surg., Gynec. and Obst.*, 1924, **39,** 62.
7. Knutti, R. E., Hawkins, W. B., and Whipple, G. H., *J. Exp. Med.*, 1935, **61,** 115, 127.
8. Queen, F. B., Hawkins, W. B., and Whipple, G. H., *J. Exp. Med.*, 1933, **57,** 399.
9. Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37,** 11.
10. Sribhishaj, K., Hawkins, W. B., and Whipple, G. H., *Am. J. Physiol.*, 1931, **96,** 449.
11. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, 1925, **72,** 395.
12. Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, 1926, **78,** 675.