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THE RELATION OF BLOOD AND TISSUE PHOSPHOLIPIDS TO EXPERIMENTAL GASTRIC ULCER

A dissertation submitted in partial
fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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by

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XVI. A PRELIMINARY REPORT ON THE RELATION OF BLOOD PHOSPHOLIPIDS TO EXPERIMENTAL GASTRIC ULCER

By
SHIRO TASHIRO and L. H. SCHMIDT*

INTRODUCTION

The discoveries that hyperthyroidism increases an animal's susceptibility to ulcer production (1) by the action of bile salts and that hyperthyroidism in itself will produce gastric ulcer (2), in view of several other facts, have certain implications. Many investigators (3, 4, 5, 6 and 7) have demonstrated the stimulating action of thyroid substance on total lipid metabolism. Since of the lipids only the phospholipids (8) and perhaps the sulfolipids (8) and cholesteryl oleate (9) have the power to inhibit bile salt ulcer production, we have suggested that the increased toxicity of the bile salts in hyperthyroidism must be due to a decrease in these antagonizers. Dudgeon (10) showed by staining methods that diphtheria toxin injected into cells produced a change in the phospholipid content of the tissues. At the same time, it has been shown by others (11, 12) that diphtheria toxin will produce gastric ulcer. These facts, coupled with the indication that both hyperthyroidism ulcers and bile salt ulcers are connected with the phospholipid metabolism, lead us to the question of the possible role of phospholipids in all forms of experimental ulcer production.

It is the purpose of this investigation to study the effect of some ulcer producing agencies on the blood phospholipids, in an effort to discover if there is a common biochemical change which may be associated with gastric ulcer production.

EXPERIMENTAL

Male rabbits were used in these experiments. They were selected in preference to guinea pigs, which we had used in our previous work, merely because of the greater ease in obtaining blood in considerable quantities from the heart. These rabbits were kept on a uniform diet of timothy hay, oats and celery. They were always starved for 18 hours before the sampling of their blood. It was not necessary to draw more than 3 cc. of blood from the heart at one time, an amount insufficient in itself (13) to produce a change in the lipids.

The blood lipid phosphorus was determined on an alcohol-ether extract (Bloor) of whole blood or plasma, according to the method of Fiske and Subbarow (14), slightly modified for our particular purpose.*

(1) *The effect of thyroid feeding on the blood phospholipids.*

Experimental hyperthyroidism was produced by feeding the rabbits varying quantities of oral thyroxin (Squibb). The animals were weighed daily, always before the feeding of thyroxin. The blood phospholipids were determined before the first feeding and 48 hours after the last feeding of thyroxin. A summary of the results of these experiments is shown in Table I. (Page 145.)

As shown in Table I, feeding of thyroxin produces a decrease in the blood phospholipids, although administration of 0.2 mgm. of thyroxin daily for four days does not produce significant changes. Administration of 2.0 mgm. or more of thyroxin to a 2 kilo rabbit over a period of five days, produces a very definite decrease in phospholipids. There was no significant change in the weight of the animals treated. Bing and Heckscher (loc. cit) found a similar relation between the total lipids and the body weight existing in the rat fed with thyroid gland. This is in marked contrast to our finding with guinea pigs where

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*A detailed description of this modification will be presented in a later publication.

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administration of 0.4 mgm. of thyroxin to a 400 gm. animal would produce a 12 — 15 per cent decrease in body weight.

In general, the phospholipid content reduction by thyroid treatment is greater the larger the initial phospholipid values. Under treatment with 2.0 mgm. of oral thyroxin, two rabbits with initial phospholipid values of 12.81 and 11.81 mgm. lost 30.68 per cent and 22.52 per cent respectively. Under the same treatment two animals with phospholipid values of 10.2 and 10.45 mgm. lost only 18.35 per cent and 11.17 per cent respectively. We may also mention that the extent of phospholipid decrease is proportional to the severity of the thyroid treatment, if the initial phospholipid values are about the same. (Compare R. 4, R. 49 and R. 95.)

In an earlier paper we pointed out that treatment with 0.4 mgm. of oral thyroxin would reduce the ulcer producing dose of bile salt for a 400 gm. guinea pig about 25 — 30 per cent. The average decrease in the phospholipids of a rabbit following treatment with an equivalent amount of thyroxin is about 20 per cent. If we dare to apply the figures for phospholipid change in the rabbit to the guinea pig, the amount of phospholipid change is very near the theoretical. For we have found that the lecithin bile salt antagonizing ratio for a normal guinea pig is approximately 0.8 : 1.0. Of course, it is quite possible that this close agreement is a mere coincidence.

(2) *The effect of adrenalin on the blood phospholipids.*

Friedman (15), Crile (16) and others have pointed out that malfunctioning of the suprarenals will result in gastric ulcer production. Bilateral and unilateral suprarenalectomy and administration of large quantities of adrenalin, both alone and with pilocarpine, produce experimental ulcer. We have therefore investigated the effect of adrenalin on blood phospholipids.

A 1:1000 adrenalin hydrochloride solution was used in all these experiments. The solution was injected subcutaneously into fasting rabbits; the blood phospholipids were determined before injection and four hours afterwards. The results are shown in Table II.

These results show that small doses of adrenalin decrease the blood phospholipids noticeably. Friedman found that a dose of 0.8 cc. of 1 : 1000

TABLE I.

Animals	Thyroxine		Body weight			Lipoidal P.		
	No. of feedings	Mgm. at each feeding	Initial weight in gms.	Final weight in gms.	% change	Initial value per 100 cc. blood mgm.	Final value per 100 cc. blood mgm.	% change
R– 3	4	0.2	2150	2100	—2.33	10.95	10.87	— 0.64
R– 4	4	0.2	2325	2325	0.00	10.13	10.41	+ 2.76
R–15	5	0.4	2650	2650	—3.51	11.81	9.15	—22.52
R–16	5	0.4	2650	2700	+1.88	12.81	8.88	—30.68
R–49	5	0.4	2400	2400	0.00	10.2	8.33	—18.35
R–51	5	0.4	2825	2750	—2.65	10.45	9.32	—11.17
R–95	5	0.6	1825	1825	0.00	9.9	7.78	—21.6
R–98	0	0.0	1625	1800	+0.76	9.61	10.26	+ 6.78
R–52	0	0.0	1650	1675	+1.51	10.27	10.41	+ 1.37
R–5	0	0.0	2325	2325	0.00	11.54	11.54	0.00

TABLE II.

Animal No.	cc. of adrenalin hydrochloride given per kilo body weight	Initial mgm. Lipid P. per 100 cc. whole blood	Final mgm. Lipid P. per 100 cc. whole blood	% change
R–37	0.2	11.11	9.74	—12.32
R–130	0.25	11.5	9.06	—21.5
R–131	0.25	9.4	7.45	—19.7
R–128	0.00	10.9	10.9	+ 1.07
R–35	0.00	10.27	10.27	— 0.68

solution was necessary to produce gastric ulcer in a 500 gm. guinea pig. Whether larger doses of adrenalin would produce proportionally greater decreases in the blood phospholipids remains to be investigated.

(3) *The effect of diphtheria toxin on blood phospholipids.*

Enriquez and Hallion (11) and Rosenau and Anderson (12) have produced gastric ulcer in guinea pigs and rabbits with repeated injections of sub-lethal doses of diphtheria toxin. These ulcers were very slow in forming and bear very great likeness to the histological character of the human ulcer.

The minimum lethal dose of our diphtheria toxin was 0.3 cc. of 1:1000 toxin. We injected 0.1 cc. of this diluted toxin into the rabbit's ear vein on every third day and followed the animal's weight closely. When the weight stopped falling, the dose of diphtheria toxin was increased to 0.2 cc. This procedure was repeated until the rabbit died. The results are shown in Table III. The blood was sampled as usual at the begining of the experiment, and again at the time when the animal showed a steady decrease in weight.

As shown in Table III this toxin produces enormous changes in the blood phospholipids (Dudgeon has already pointed out its effect on the cell phospholipids). Both of these animals died, autopsies showing ulceration of the gastric mucosa. (Dr. Guest and Miss Ashley of the Pediatrics Department noted a similar change in their study of phosphorus partition in dipththeria intoxication.)

(4) *The effect of Streptococci on blood phospholipids.* Many investigators have studied the infectious origin of gastric ulcer. Injection of pus from a general pyemia (17), (18), of Staphylococci and Streptococci (19), (20), and injection of Pneumococci (21) will all produce gastric ulcer under the proper conditions.

In the experiments only two strains of Streptococci were investigated—*viridans* and *pyogenes*. They came from stock cultures, the origin of which was not known at the time. They were grown in tubes containing dextrose ascites broth for 24 hours. At the end of that time, the tubes were centrifuged and the supernatant liquid decanted, the Streptococci suspended in saline and injected into the ear vein of a rabbit. The blood was sampled before the injection and again as soon as the animal became noticeably ill. Both of the animals died; one (R. 73) in 3.5 hours and the other (R. 75) in 11.75 hours after the final heart punctures. In both cases, immediate autopsies revealed numerous lesions of the stomach mucosa. The results of analyses are shown in Table IV.

TABLE III.

Exper. No.	cc. of dilute toxin administed'	Days duration of expts.	Initial weight in gms.	Final weight in gms.	% change	Initial mgm. Lip. P. per 100 cc. whole blood	Final mgm. Lip. P. per 100 cc. whole blood	% change
R–31	0.3	10	1850	1450	21.63	12.1	8.88	—26.62
R–32	0.7	24	2275	1800	20.9	13.32	6.52	—51.2

TABLE IV.

Animal	Strain of Streptococcus injected	Initial Lip. P. per 100 cc. whole blood	Final mgm. Lip. P. per 100 cc. whole blood	% change
R–75	pyogenes	8.36	6.14	—26.5
R–73	viridans	9.96	8.84	—11.2

The phospholipids of both of these animals decreased considerably. It would be rash to generalize as to the effect on the blood phospholipids of all the infections that produce ulcerations. It may be interesting, however, to add that one strain of Streptococci was found to increase the blood phospholipids and this same strain made guinea pigs more resistant to taxicity of the bile salts (22).

(5) *The effect of bile salts on the blood phospholipids.*

Sellard's discovery of the ulcer producing action of bile and bile salts (23) has been repeatedly

confirmed in our laboratory. For the purpose of considering whether bile salts or phospholipids form the common basis of gastric ulcer production the question of the effect of bile salts on the phospholipids of the blood has been carefully investigated.

The bile salts used in this series of experiments were a mixture of the sodium salts of taurocholic and glycocholic acids (Fairchild Bros. and Foster). They were administered in physiological saline solution both intravenously and intraperitoneally.

Since in previous experiments we have injected the bile salts intraperitoneally, we first used this method of injection in studying the effect of varying quantities of bile salt on the blood phospholipids. The blood was drawn before injection and five hours after injection. The controls were injected with 2.5 cc. of normal saline. The results are shown in Table V.

Table V shows that intraperitoneal injection of bile salt produces decreases in the blood phospholipids. If these decreases are plotted against the doses of bile salts, the curve is a fairly straight line, as shown in Figure 1.

It must be remembered that the amounts of bile salt administered here varied from 12 to 37 per cent of the minimum ulcer producing dose for a rabbit (0.175 gm. per kg. body weight). We are investigating the effect of large doses at the present time.

These animals injected intraperitoneally with bile salts were greatly excited—much more so than the controls. This suggested that the decrease in phospholipids might have been produced by suprarenal hyperactivity, due to the irritating action of the bile salt within the peritoneal cavity. To test this suggestion several animals were injected intravenously with the results shown in Table VI, and Figure 1. (Pages 147, 149.)

It is evident that the action of bile salts on the

TABLE V.

Animal No.	Gm. bile salt per kilo wt.	Initial mgm. Lip. P. per 100 cc. whole blood	Final mgm. Lip. P. per 100 cc. whole blood	% change
R–8	0.02	12.92	11.36	—12.07
R–9	0.02	12.3	10.79	—12.27
R–11	0.04	13.29	10.71	—19.14
R–12	0.04	13.27	11.71	—11.7
R–19	0.06	11.71	9.2	—21.44
R–20	0.06	11.9	9.61	—19.19
R–10	0.00	11.71	11.9	+ 1.6
R–14	0.00	12.0	12.2	+ 1.7
R–21	0.00	8.82	8.57	— 2.9

TABLE VI.

Exper. No.	Gm. bile salt per kilo wt.	Hours before 2nd sampling	Initial mgm. Lip. P. per 100 cc. whole blood	Final mgm. Lip. P. per 100 cc. whole blood	% change
R–180	0.06	4	11.6	9.3	—20.0
R–186	0.02	3	9.6	9.69	— 9.5
R–188	0.04	3	9.34	8.08	—13.5
R–190	0.02	2	9.64	9.0	— 6.64
R–192	0.06	2	9.8	8.0	—18.5

TABLE VII.

Exper. No.	Amt. bile salt per kilo body wt.	Initial mgm. Lip. P. per 100 cc. plasma	Final mgm. Lip. P. per 100 cc. plasma	% change
R–179	0.06	3.88	4.08	+5.1
R–187	0.02	2.25	2.15	—4.65
R–189	0.04	2.1	2.15	+2.37

phospholipids is not due to their irritant action within the abdominal cavity. It is again apparent from Curve B in Figure 1 that the decrease in blood phospholipids is proportional to the amount of bile salt administered. It will be noticed also that the phospholipid values in Table VI are on the whole smaller than those found in Table V. This may be explained by stating that the first group of experiments was performed in early fall and that the latter group was performed in the late winter. These findings are in accord with the findings in our other experiments, although the reverse of the finding in the experiments with amphibians. There is a gradual decrease in the phospholipid content of rabbit blood during the winter months and an increase during the latter part of the spring.

In the previous experiments the phospholipid values were determined on whole blood. It is known that the cholesterol content of the corpuscle is nearly constant and it was thought that the phospholipid content might also possess that property. It is also known that the phospholipid content of the plasma is much less than that of the corpuscle; so that in order to determine small plasma phospholipid changes one must use plasma alone for analysis. We have investigated the effect of bile salt injection on plasma phospholipids with typical results shown in Table VII. (Page 147.)

From the results of this table one can only conclude that the change in phospholipid content of the blood produced by bile salt injection takes place not in the plasma but in the cell. An investigation is now in progress to determine the fate of the lipid phosphorus lost by bile salt treatment.

DISCUSSION

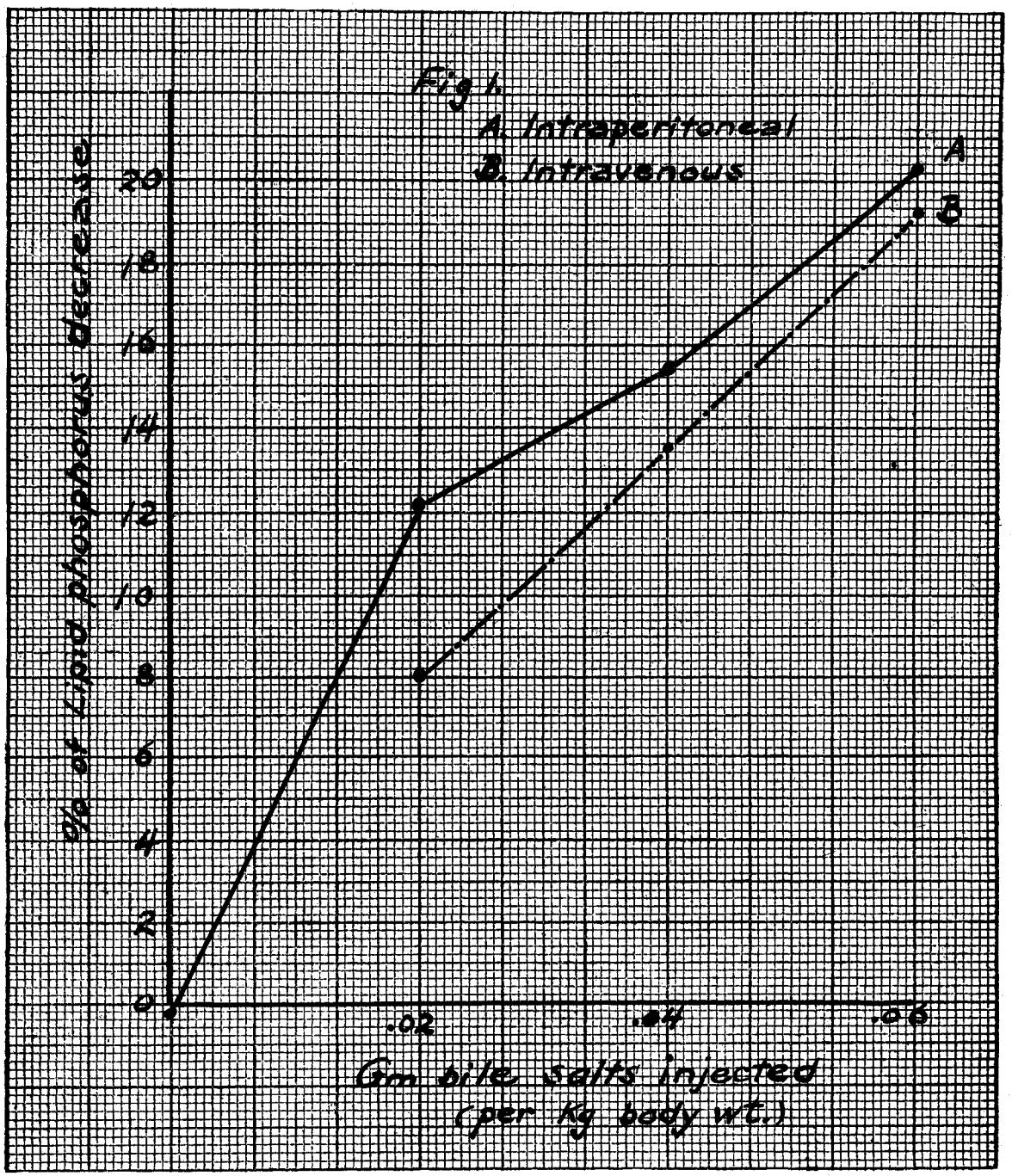
It is evident from the results of these experiments that treatment by all methods we have yet employed which are likely to produce gastric ulcer involves a simultaneous decrease in phospholipids. Since these phospholipids, which are the most effective bile salt antagonizers, also decrease in amount under the action of bile salts, a question arises whether bile salts are but one of several gastric ulcer forming agencies, or all the other agencies work through bile salts. In other words, is the decrease in phospholipids *per se* responsible for gastric ulcer formation?

The nature of the different body phospholipids and their specific functions is still obscure. But there seems to be no question that one of their general functions is to maintain certain physical and physico-chemical conditions at a certain level. And all the tissue phospholipids are no doubt kept in a delicate balance with the blood. Thus, if the decrease in phospholipids is alone responsible for the gastric ulcer formation, we might imagine that a certain fraction of the cell phospholipids may be concerned with protection of its own tissue against proteolytic and other ferments as suggested by others. When there is an appreciable reduction in these protective phospholipids, through any of the agencies, the typical lesion may result. For the consideration of this possibility, it is important to know whether or not there is a considerable decrease in the phospholipid content of the stomach tissues under this condition, and if so, whether or not such a decrease would accelerate the production or activity of autolytic and other hydrolytic enzymes.

On the other hand, it must be noted that although we have found that bile salts behave as other agencies in respect to the phospholipids, we must again emphasize the fact that a phospholipid decrease increases the toxicity of the bile salts. We have also noted that in the case of diphtheria intoxication a 26.5 per cent decrease in phospholipids accompanies gastric ulcer production, while in Streptococcus intoxication the decrease was only 11 per cent when ulcer occurred. On the other hand, decreases of 20 to 30 per cent occurred with adrenalin, thyroxin and bile salt without any evidence of ulcer. This may mean either that there is a particular fraction of the phospholipids that must be reduced to produce gastric ulcer—the fraction being reduced by the Streptococci when the other fractions are untouched—or that there is something other than the phospholipid change which is responsible for ulcer formation.

It is well known that there is a close relationship between thyroid and adrenal. It has recently been shown that hyperplasia of the thyroid gland may result from either diphtheritic or Streptococcic intoxication (24), (25). It is quite possible that here again we may have the bile salts poured into the blood stream as a result of some action of the thyroid on the liver, or if the bile salts are normally present in the blood, their toxicity will be aggravated by the hyperactivity of thyroid.

This is only a preliminary investigation. Be-



fore arriving at any conclusion, it is necessary to see if there is any condition where an appreciable decrease of phospholipids can occur without gastric ulcer. The final answer to this problem will be reached more quickly once we have determined whether or not normal blood contains bile salt or whether the blood of gastric ulcer patients contains bile salts.

CONCLUSION

The effects of treatment by gastric ulcer producing agencies on the blood phosphatid content of rabbits are as follows:

(1) Oral administration of 0.2 mgm. of thyroxine daily for four days does not produce significant changes, but that of 0.4 mgm. for five days causes a decrease of 11.17 per cent to 30.08 per cent.

(2) Subcutaneous injection of 0.2 to 0.25 cc. of adrenalin hydrochloride (1:100) causes a decrease of 12.32 per cent to 21.5 per cent.

(3) Intravenous injection of 0.1 cc. of 1:100 diptheria toxin three times at three-day intervals causes 26.62 per cent reduction; after seven injections, 51.2 per cent.

(4) Intravenous injection of a Streptococcus pyogenes suspension caused 26.5 per cent decrease, and that of S. viridans 11.2 per cent.

(5) Both intraperitoneal and intravenous injections of bile salts in sublethal doses caused 6.64 per cent to 21.44 per cent decrease, the extent being proportional to the dosage, and also to the initial phospholipid content. This decrease is due to that of cell phospholipids, not those of plasma.

(6) Thus, it appears that a decrease of blood phospholipids is a condition which occurs in all forms of the treatments that are known to produce gastric ulcer in animals.

(7) Whether experimental gastric ulcer is produced by phospholipid decrease *per se* or by aggravating the action of bile salts as the consequence of such decrease, is not yet definitely settled.

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XVII. THE EFFECT OF THE ADMINISTRATION OF BILE SALTS ON PETTENKOFER POSITIVE SUBSTANCES AND LIPID PHOSPHORUS CONTENT OF BLOOD.

(Preliminary Report)

By
SHIRO TASHIRO and L. H. SCHMIDT

In a previous experiment, one of us has found that the feeding of bile salts to a patient having a high content of "blood salts"* greatly reduced the amount of the latter substances (1). Since this patient had a definite symptom of hepatic insufficiency, and since it is believed that a bile salt constitutes the greater part of "blood salts," this finding led him to propose the hypothesis that if bile salts were ingested by a person having a high "blood salts" value, the liver would be stimulated to secrete bile salts, thereby removing a part of the blood salt out of the blood. According to this hypothesis, the decrease of "blood salts" on feeding of the bile salts is due primarily to the decrease of the bile salt in the blood. If the liver is efficient and the amount of "blood salts" is normal, then ingestion of bile salts might cause an increase in the "blood salts" content. In order to test this hypothesis, a series of experiments was undertaken.

Since it has been shown that phospholipids play some part in producing the color reaction of the "blood salts" (2), and since the blood phospholipids of rabbits are decreased by injections of bile salt (Tashiro and Schmidt, 3), the changes in the phospholipid content of the blood were also followed during the course of these experiments.

These experiments were made with human subjects. Although the number of the subjects we used was not great enough to allow us to draw any positive conclusions, the results were so uniform and interesting that we shall present them here as a preliminary report.

*These Pettenkofer positive substances in blood are called "blood salts" as usual.

EXPERIMENTAL

Seven normal individuals and three pathological cases were selected for these experiments. These pathological cases were selected from those to whom we believed the administration of bile salts would be beneficial. Of normal subjects, the first two cases (B. S. 1 and B. S. 2, both males) prepared their own meals together in the laboratory and the last three girls kept house together. Subjects 4 and 5 were on a free diet. One of each group was selected as a control, and to the rest, varying amounts of bile salts were given. Of the pathological group, no control was chosen, and the diet was free as far as the physician's orders were concerned.

The bile salts ingested were a mixture of the sodium salts of taurocholic and glycocholic acids (Fairchild Bros. and Foster). A 6.5 per cent aqueous solution was prepared and kept in the ice box and the prescribed amount of this was taken one hour after each meal for fourteen days. The prescribed doses varied from one and one-half to three grains daily.

The samples of blood were drawn two hours after breakfast on the day the treatment was to start, and after fourteen days of treatment, they were again drawn two hours after breakfast, the same meal being taken on each day of drawing of blood. The "blood salts" determinations were made according to the Tashiro method (4). The lipid phosphorus was determined on an alcohol-ether extract of the blood, according to a modification of the method of Fiske and Subarrow (5).

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RESULTS

The results of these analyses are given in Table I and II. Table I shows the results of experiments on the healthy subjects, and Table II shows those obtained by feeding of bile salts to the pathological individuals with symptoms of biliary disorders. Graphic representations of these data are given in Figure 1. The solid black blocks indicate the values obtained before the administration of bile salts, and the blank blocks indicate those after two weeks' feeding of bile salts. The results of controls therefore are expressed by two solid black blocks, for both initial and final values.

There results are extremely interesting. In the first place, the controls (B. S. 2 and B. S. 6) showed practically no change either in "blood salts" or lipid phosphorus after fourteen days.

The results of feeding bile salts, however, present two distinct pictures in the normal individual. B. S. 1 and B. S. 7 were persons with normal "blood salts" content. Feeding of three grains daily to the former and one and one-half grains daily to the latter produced considerable increases in the "blood salt" content. On the other hand, B. S. 4, B. S. 5, and B. S. 8 were persons with high initial "blood salt" contents. Administration of one and one-half grains of bile salts daily to B. S. 4 and B. S. 8 resulted in substantial decreases; while that of three grains daily to B. S. 5 caused a very slight decrease.

TABLE I.

Normal.

Diet	Subject	Grains of bile salt administered daily	Mgm. blood salt 100 cc. of blood		Mgm. Lipid Phos. 100 cc. of blood	
			Initial value	Final value	Initial value	Final value
Same Diet for Both	B. S. 1	3	136	196	12.2	11.1
	B. S. 2	0	136	134	11.2	11.1
Free Diet	B. S. 4	1½	236	142	11.3	10.1
Free Diet	B. S. 5	3	188	175	12.7	11.1
Same Diet for all three	B. S. 6	0	140	149	12.3	12.3
	B. S. 7	1½	152	247	12.6	11.3
	B. S. 8	1½	222	147	12.0	11.2

TABLE II.

Pathological.

Diet	Subject	Grains of bile salt administered daily	Mgm. blood salt 100 cc. of blood		Mgm. Lipid Phos. 100 cc. of blood	
			Initial value	Final value	Initial value	Final value
Free	G. C. 13:21	3	102	185	16.2	12.4
Free	G. C. 15:25	1½	135	152	13.1	13.9
Free	G. C. 14:26	1½	164	135	12.1	13.7

As far as the normal individual is concerned, therefore, our results show that the effect of bile salt feeding on "blood salts" depends upon the initial "blood salts" content. When the content is high, bile salts tend to diminish it; and when it is low, bile salts tend to raise it.

The effect on lipid phosphorus is slightly different from that on "blood salts." The administration of bile salts decreased phospholipids in all healthy subjects, decreases ranging from 0.8 to 1.6

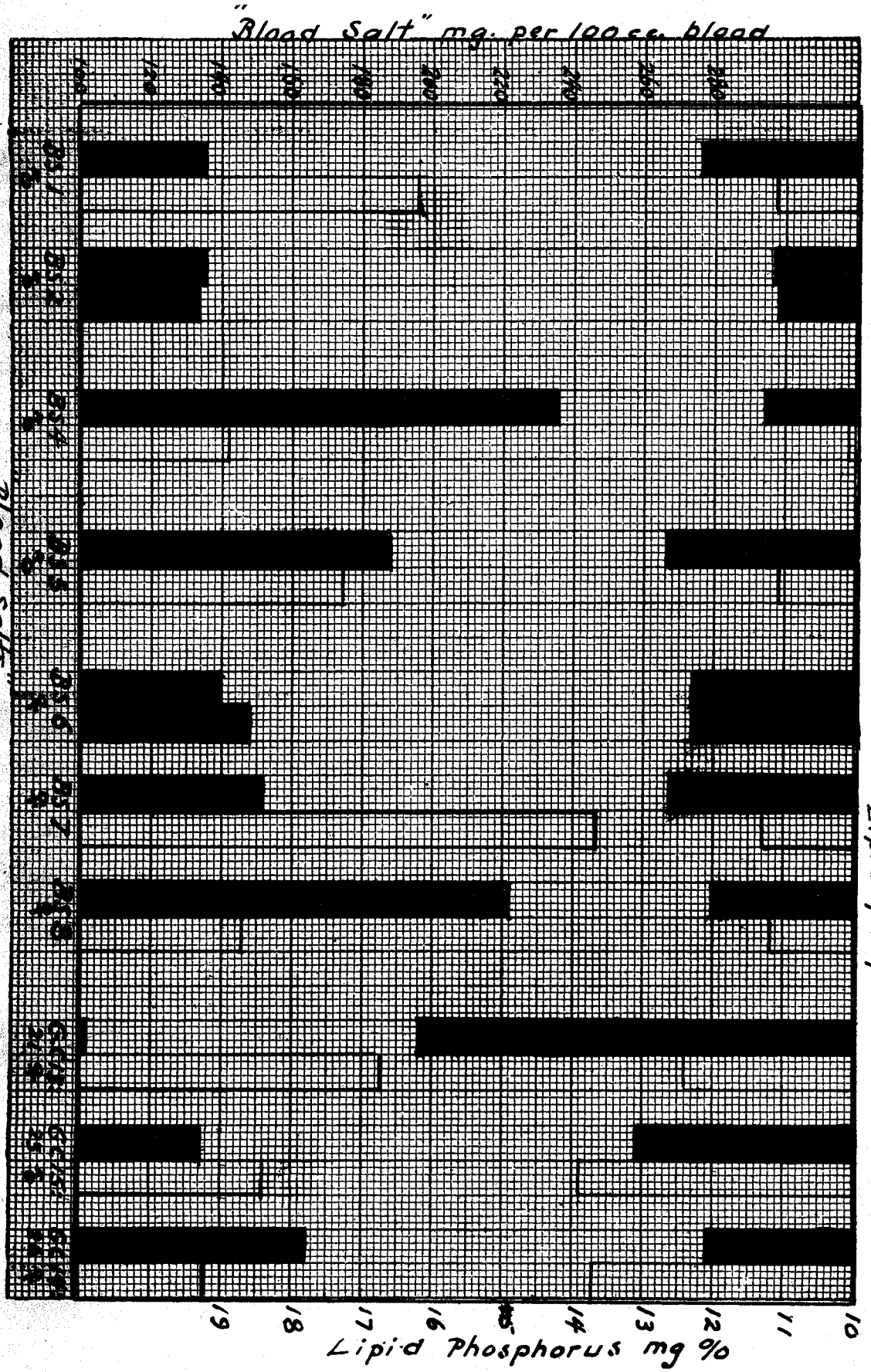

Fig. 1

mg. per cent as phosphorus (approximately around 10 per cent decrease). This is quite in keeping with our results in the case of the decreased blood lipid phosphorus following intraperitoneal or intravenous injection of bile salts into rabbits (3). These results show, incidentally, that phospholipids cannot be wholly responsible for the Pettenkofer color of "blood salts" in spite of the fact that almost all (94 per cent) of the phospholipid goes into our alcohol-acetone filtrate. Since the decrease in the "blood salts" occurs only when the initial "blood salts" is high, while that in phospholipids takes place in every normal case, a parallel change between these two does occur whenever bile salts are administered to a subject having a high initial "blood salts." But even in this case, the decrease in phospholipids is too small to account for the decrease of "blood salts" according to our results obtained in connection with the color values of phospholipids in Studies III.

Of the pathological cases (Table II), G. C. 13:21 was a woman suffering from intermittent attacks of jaundice and an undiagnosed gastro-intestinal disorder. Her initial "blood salts" content was very low and her blood phospholipid content very high. Treatment with three grains of bile salts daily for fourteen days produced an increase in "blood salts" and a decrease in the phospholipids. G. C. 15:25 was a man suffering from symptoms of hyperthyroidism and not infrequent attacks of jaundice. Administration of one and one-half grains of bile salts to this man daily for fourteen days increased his "blood salts" content, but also produced an increase in the blood phospholipids. G. C. 14:26 was a woman suffering from an undiagnosed gastro-intestinal disorder and had a slightly high initial "blood salts" content. Administration of bile salt caused a decrease in the "blood salt" content and an increase in blood phospholipids.

Thus in pathological cases, too, the effect of bile salt feeding on "blood salts" seems to be determined by the original "blood salts" content. Although the number of experiments is too few to give an exact critical line which divides these effects into two directions, we note 160 mg. per 100 cc. blood to be the approximate critical value. In both normal and pathological cases, all those whose initial "blood salts" values were above this, lost "blood salts" on the bile salts feeding; and those below this, gained "blood salts" after exactly the same treatment.

The effect of bile salt administration on the blood phospholipids of pathological cases is not as uniform as we found in normal subjects. In one case whose initial lipid phosphorus value was very high, the feeding caused a diminution of 3.8 mg. phosphorus. In the other two cases, the feeding raised the lipids to an appreciable degree, in spite of the fact that initial values were equal to or slightly more than those of the normal subjects. If it were not for the fact that the original phosphorus values were not below normal, these increases might easily be attributed to an improvement in absorption of dietary fat following the administration of bile salts. Whether the phenomenon of an increase of phospholipids upon bile salt feeding is characteristic of certain hepatic diseases or is a mere coincidence, has to be determined by further experiments with a more extensive series of pathological cases.

As far as "blood salts" are concerned, our hypothesis that the normal blood salts should increase on bile salts feeding has proven to be correct. But this fact does not prove the correctness of our original assumption that a bile salt constitutes the greater part of blood salts. For this reason, any consideration as to the reason why the administration of bile salts affects "blood salts" will remain a mere opinion as long as the nature of all "blood salts" remains unproven.

We have already indicated that these changes in "blood salts" content following the administration of bile salts could not be wholly due to changes in phospholipids. Could they be due to changes in cholesteryl oleate? Unfortunately we could not determine the changes of this interesting compound because of the lack of a method.

There is, however, a report by Gardner and Gainsborough (6) on the effect of bile salts feeding on cholesteryl esters in hepatic cases. According to their finding, cholesteryl esters are increased on the administration of bile salts, the increase being usually within 100 mg. and mostly far less, except when the original ester value was exceedingly small. It seems therefore possible that some of the "blood salts" increase by bile salts feeding may be due to the increase of cholesteryl oleate. Quantitatively speaking, however, such an increase of bile salts as we find cannot be wholly explained

by that of cholesteryl oleate. Even if we may assume that all of the ester were the oleate, an increase of 100 mgms, could produce but a fraction of the color increase observed (cf. Studies III). The case of decreased "blood salts" content upon bile salt feeding is more difficult to attribute to a change in cholesteryl oleate. According to these investigators a decrease in cholestryl esters occurs when an abnormal decrease in fat absorption produces a demand on the fatty acid part of the ester. As far as the fat absorption is concerned, it is very difficult to see how the feeding of bile salts can cause such a demand on cholesteryl esters. Yet, when blood salts were present in amounts more than 160 mg. per cent, we found a decrease of "blood salts" on bile salts feeding. Certainly cholesteryl oleate cannot be wholly responsible for every change of "blood salts."

In this connection it is interesting to note that subject B. S. 1 showed practically a quantitative increase in the "blood salts" value after ingestion of bile salts. During the fourteen days of the experiment, he took 2.73 gm. of bile salt. Regardless of the nature of the "blood salts" initially he has had these substances in amounts equivalent to 6.53 gm. of bile salt. And at the end of fourteen days, his "blood salt" increased to the amount equivalent to 9.41 gm. of bile salts. The actual increase in Pettenkofer positive substance in the blood by the administration of 2.73 gm. bile salt was equivalent to 2.88 gm. bile salts (9.41-653 gm.). No doubt, this close agreement is only a coincidence, because the other individuals did not show such quantitative increases; besides the assumption that all the ingested bile salts might find their way into the blood, and be retained there for fourteen days, is not in accord with the current understanding of "circulation" of bile salts.

Whatever may be the nature of "blood salts," and the mechanism of their changes following the administration of bile salts, we believe that the feeding of bile salts is equally beneficial for those who have high "blood salts" and those who have low "blood salts." Meanwhile, the interpretation of these interesting actions of the bile salts on "blood salts" as well as on phospholipids, must await further experimental data.

CONCLUSION

(1) In both normal and pathological cases, all those who had "blood salt" above 160 mg. per 100 cc. blood, lost some of "blood salt" when they took bile salts for fourteen days, and those who had below that amount, gained it.

(2) In all normal cases, lipid phosphorus decreased when the bile salts were given by mouth for fourteen days.

(3) In pathological cases, the effect of phospholipids was not uniform.

(4) As far as two normal controls were concerned, both "blood salts" and lipid phoshorus were found practically the same after fourteen days.

The authors wish to take this opportunity to acknowledge their gratitude to the following individuals for their co-operation and assistance in carrying out these experiments: Dr. Genther, Dr. Wiley, Mr. M. Boyd, Mr. D. Irish, Mr. E. Dunn, Mr. E. Adams, Miss A. Ashley and Miss C. Hogden.

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III. QUANTITATIVE STUDIES ON THE PETTENKOFER REACTION OF DIFFERENT LIPIDS AND OF BLOOD FILTRATE.

By

SHIRO TASHIRO, L. H. SCHMIDT and ESTHER BOGEN TIETZ

INTRODUCTION

In devising the method (1) for determination of Pettenkofer positive substance in blood (which will be designated as "blood salts," as usual), one of us had the idea in mind that if normal blood contains a bile salt, it must be that of deoxycholic acid, and that if this salt were present, any method of estimation of bile salts in blood should not be so exclusive as to miss this acid. Such a method would necessarily include other Pettenkofer positive substances, for the physical and chemical properties of deoxycholic acid are, in many respects, more nearly like those of other Pettenkofer positive compounds than are those of cholic acid.

In order, therefore, to form some quantitative conception as to the probable presence of a bile salt from the results obtained by our method, it is very necessary to make a quantitative study of the Pettenkofer reaction of other compounds, and see how much each of these contributes toward the total color obtained from "blood salts."

Among the substances most likely to interfere with our method are the phospholipids, or particularly lecithin and cephalin. In spite of the fact that these two substances are normally present in appreciable quantities, our method deliberately omits any device to eliminate them, on account of the fact, as already pointed out, that absolute separation of these substances from bile salts cannot be effected by ordinary solvents. According to Walker (2), cholesteryl oleate may also be added to the list of substances found in blood that are strongly Pettenkofer positive.

As will be shown later, strangely enough, these three compounds have the power of neutralizing certain toxic actions of bile salts (3, 4), the fact making the study of their Pettenkofer reaction of more than passing interest.

The present investigation was undertaken, therefore, to determine the color values of lecithin, cephalin and cholesteryl oleate, primarily to determine the extent of interference of these compounds in our method, and incidentally to see if certain peculiar reactions of blood filtrates that we observed in previous studies (5) should be attributed to these compounds rather than to deoxycholic acid. As an addendum, we shall include in this report an easy method of distinguishing deoxycholic acid from cholic acid by means of ultraviolet fluorescence.

EXPERIMENTAL

The Pettenkofer reaction of lecithin, cephalin and cholesteryl oleate was studied with and without the blood filtrate.

Eastman lecithin (practical) was used in these experiments. This particular preparation contained 2.5 per cent alcohol—insoluble material. A 0.05 per cent solution of this substance in absolute alcohol was used, only the clear portion of the solution being taken for the determination. (In calculating the percentage of the solution we ignored this 2.5 per cent error.)

A 0.05 per cent ether solution of cephalin prepared by one of us (S) was used in the cephalin experiments. Although repeated efforts to purify this substance were made, the final product was still impure (the P:N ratio = 1.12:1.0).

A 0.05 per cent alcoholic solution of cholesteryl oleate was used in these experiments. The ester was prepared for us by Dr. K. Ishii, according to the Hürthle method (6). The product used had a m. p. of 38° C.

Two different alcoholic acetone filtrates were used; one was an extract of whole dog blood, and the other an extract of horse blood.

The color values of these lipids and the filtrate,

Submitted by L. H. Schmidt to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1932.

under various conditions, were estimated by the Tashiro method, using the mixed salt of taurocholic and glycocholic acid (Fairchild Bros. and Foster) as a standard; wherever the lipids were added to the filtrate, the acetone treatment for removal of cholesterol was applied after the addition.

RESULTS

(A) The mixed salts alone.

The color value of varying concentrations of the mixed salts has already been determined by one of us using a 0.25 per cent standard (1), and again with a 0.03 per cent standard (2). But as directed in the method since a use of a 0.02 per cent standard was found more suitable for all the analyses, we found it necessary to redetermine it with this standard. The results of such determinations with 0.04 per cent, 0.06 per cent, 0.08 per cent, 0.10 per cent and 0.12 per cent are given in Table I and Figure 1. The color values are expressed as per cent of the color of 0.02 per cent standard. (See Figure 1, page 64.)

TABLE I.

The color value of varying concentrations of bile salt. Standard: 0.02%

Conc. Mixed Salts	Found calc'd as % Standard	Taken as % of Standard	% Deviation from Theoretical
.04%	192.5	200	— 3.75
.06%	325.0	300	+ 8.33
.08%	444.0	400	+11.00
.10%	617.0	500	+23.4
.12%	933.0	600	+55.5

The results shown in this table are quite in accord with the theoretical value until the ratio of the concentration of the unknown to that of the standard exceeds four. At that point, that is, when the unknown is four times as concentrated as the standard, there is a rapid increase in the color value until at a ratio of six, the unknown produces 55 per cent more color than the theoretical. As will be brought out in the next paragraph, this excess color producing power of the mixed bile salts (or of cholic acid as stated in studies II), is in striking contrast to that of the lipids. In this respect deoxycholic acid alone behaves similarly to these lipids and to blood filtrate.

Thus the results given in Table I are also quite in accord with those obtained before, namely the mixed salts give a greater color value than the theoretical when determined with a weaker standard. The per cent of deviation from the theoretical in this series is not as great as those found with a higher standard. It may be recalled that with a 0.025 per cent standard, 0.06 per cent bile salts gave 21 per cent more color than the theoretical; with a 0.03 per cent standard, the same unknown solution gave 16 per cent more, while with our weak standard (0.02 per cent), a 0.06 per cent unknown mixed salt gave only 8.33 per cent more color than the theoretical.

(B) The color value of lecithin.

The color value of varying concentrations of lecithin was determined under three conditions.

(1) Lecithin alone.

The color value of 0.02 per cent-0.14 per cent lecithin solution was compared with a 0.02 per cent standard (the mixed salts). The results are given in Table II in which the color value of each lecithin solution is expressed as per cent of the color value of the standard as usual. The color increase per 0.02 per cent increment is also expressed on the basis of the color of the standard.

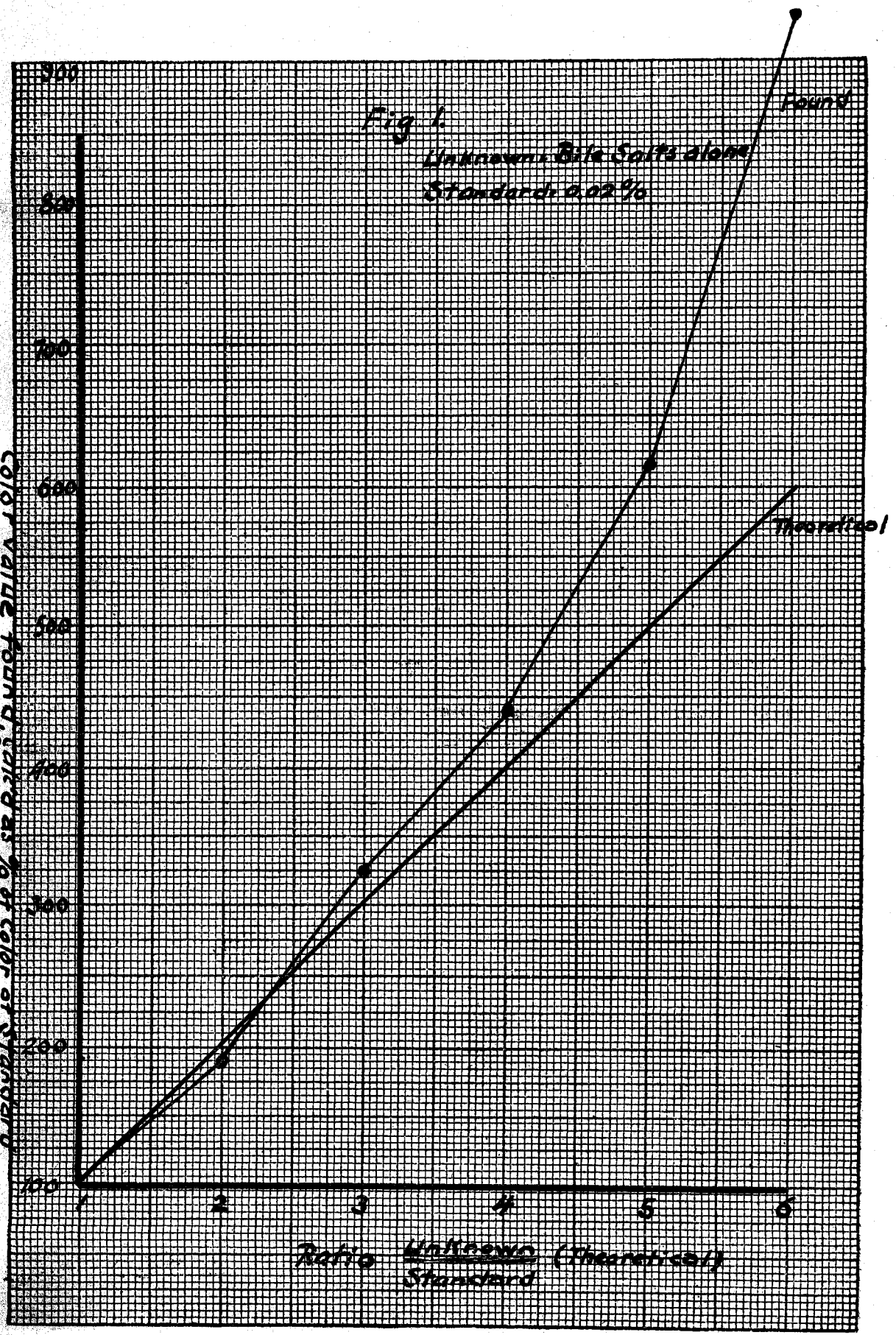
TABLE II.

The color value of varying conc. of lecithin. Standard: 0.02% *mixed salts.*

Conc. of Lecithin %	Found calculated as % Standard	Increase per 0.02% Lecithin
0.02	45.95	
		5.45
0.04	51.4	
		8.9
0.06	60.3	
		7.6
0.08	67.9	
		6.7
0.10	74.6	
0.12	?	
		7.6
0.14	97.8	

As shown in Table II, the increase of the color value of lecithin solution alone is quite uniform, the average being 7.2 per cent of the standard for each 0.02 per cent increment. From the table, it is also quite plain that the color value of lecithin is about 2/15th of that of the mixed salt. Even the color of a 0.14 per cent lecithin solution is not quite equal to that of a 0.02 per cent mixed salts solution.

If normal human blood contains roughly 200 mgm. of lecithin per 100 cc., its color value will be equivalent to about 26 mg. of the mixed salt. Since



our acetone alcohol mixture extracts only about 94 per cent of total phospholipids (Miss Spencer, 7), approximately 25 mgm. of the blood salts must be due to lecithin. However, the presence of blood constituents modifies the color of many Pettenkofer positive substances (5), and the color value of lecithin might be modified when added to the blood filtrate.

(2) Lecithin and blood filtrate.

In these experiments, we repeated the analysis of the same concentrations of lecithin as before, except that each solution contained 5 cc. of horse blood filtrate (0.182 cc. of the original sample). This filtrate alone as a control had the color value of 67.4 per cent of the standard. The results are given in Table III.

TABLE III.

The color value of lecithin when added to the blood filtrate)5 cc. A : A). Filtrate had color value 67.4% standard. Standard: 0.02% mixed salts.

Conc. of Lecithin %	Found calculated as % Standard	Increase per 0.02% Lecithin
0.02	81.6	14.2
0.04	96.2	14.6
0.06	112.2	16.0
0.08	127.0	14.8
0.12	135.5	4.3

It is plain from Table III that the rate of the color increase of lecithin becomes greater when the blood filtrate is added. In spite of the fact that each solution has exactly the same amount of the blood filtrate, the same increment of lecithin produces about twice as much color as in the case of lecithin alone. This fact does not mean, however, that the color value of lecithin in the presence of the filtrate is twice as great as that of lecithin alone, as the following calculation shows:

If normal human blood contains roughly 200 mgm. of lecithin per 100 cc., 94 per cent of it will go into our acetone alcohol filtrate, that is 188 mgm. Since for the final analysis the blood is diluted by 5.5 times, the final lecithin concentration should be $\frac{0.188}{5.5}$ per cent or 0.034 per cent. According to our analysis, each 0.02 per cent lecithin in the presence of the filtrate will increase the color by 14.4 per cent of the standard (0.02 per cent), so 0.034 per cent lecithin should increase the color 24.5 per cent of 0.02 per cent bile salts. Since .02 per cent bile salt is equivalent to 110 mg per 100 cc. (.02 gm X 5.5), 24.5 per cent of 110 mg is 27 mg. Thus 200 mg of lecithin will give the color value equivalent to that of 27 mg. bile salts. So in normal blood, containing 200 mg per cent lecithin, 27 mg per cent of the "blood salts" can be attributed to lecithin alone.

It should be noted that when the lecithin concentration is more than 0.08 per cent (four times concentration of the standard), the color value no longer increases as much, the same change in concentration producing only 4 per cent of the standard. This is probably due to the fact that when a large amount of lecithin is added to the filtrate, scums are formed which are colored intensely purple. Since we did not find any scums with lecithin alone, within the range of concentrations used, it might be possible that union occurs between lecithin and one of the constituents of the filtrate. We have consequently tried a combination between lecithin and bile salts.

(3) Lecithin and the mixed salt.

The experiments were exactly the same as before, except that each lecithin solution contained 0.02 per cent bile salts. The color values obtained from these analyses are given in Table IV.

TABLE IV.

The color value of lecithin when added to 0.02% mixed salts. (0.02% mixed salt as standard).

Conc. of Lecithin %	Found calculated as % Standard	Increase per 0.02% Lecithin
0.02	89.2	—10.8
0.04	103.2	14.0
0.06	124.5	21.3
0.08	150.2	25.7
0.10	?	
0.12	?	2.7
0.14	158.2	

Two facts should be noted from Table IV. In the first place, an addition of 0.02 per cent bile salt to 0.02 per cent lecithin gave less color than 0.02 per cent bile salt alone. Why an addition of a small amount of lecithin causes a loss in the color is very difficult to explain unless we assume some combination between them. There is also a tendency for the mixture to give more increased color per 0.02 per cent increment as the concentration increases

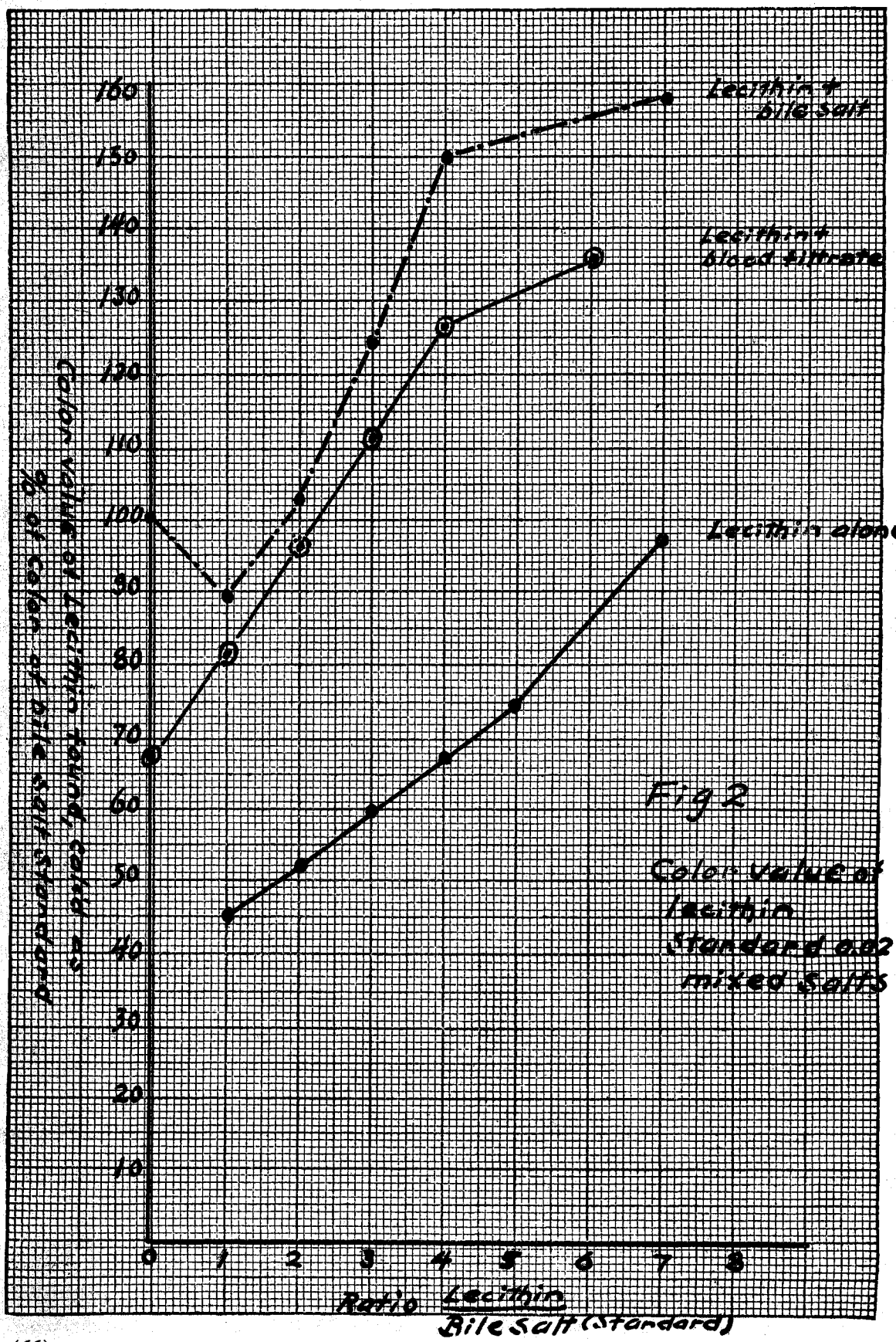


Fig 2

(66)

until, at 0.08 per cent lecithin, the curve suddenly bends, showing the color approximately approaching a maximum. This fact is similar to that which we observed with the mixture of lecithin and blood filtrate. It is interesting to note that around these concentrations we also found scum formation. This similarity suggests the possibility that the scum formation observed with lecithin and the filtrate may be due to a union between lecithin and a bile salt in the filtrate. At any event, the fact that scums are formed with these mixtures and not with lecithin alone is very significant whatever may be the reason. It is very unfortunate that we have as yet no data with mixtures of lecithin and deoxycholic acid. The results of all lecithin analyses are shown in Figure 2, on page 66.

(*C*) *The color value of cephalin.*

(1) Cephalin alone:

Cephalin, another of the interfering substances, gives the most intense color of all those substances we have investigated. A comparison of Table V and Figure 2 shows that the values are from 10 to 35 per cent higher than the color values of similar concentrations of lecithin. Since the presence of the blood filtrate modifies the color value of these lipids, we shall not attempt to calculate from these data the amount of the color cephalin contributes to "blood salts."

TABLE V.

The color value of cephalin (.02% bile salt as a standard).

Conc. of Cephalin %	Found calculated as % Standard	Increase per 0.02% Cephalin
0.02	52.9	
		9.4
0.04	62.3	
		6.6
0.06	68.9	
		28.3
0.08	97.2	
		15.1
0.10	112.3	
		6.0
0.12	118.3	
		16.4
0.14	134.7	

(2) Cephalin and blood filtrate.

A combined blood filtrate obtained from dogs (Studies V), was used which had a high "blood salts," the color value being equivalent to 111.7 per cent of the standard. The results of the analyses of these mixtures are given in Table VI.

For some reason or other, the color increase per 0.02 per cent cephalin is not uniform in both the case of cephalin alone and of cephalin with the filtrate. For this reason, the calculation may not be exact. According to Nakamura (8), normal human blood contains 95-110 mgm. cephalin per 100 cc. Since 94 per cent of total lipid phosphorus goes into our filtrate, as we stated before, we may consider roughly 100 mgm. to be the maximum for normal blood. This amount of cephalin would give us 0.0182 per cent cephlin in the final solution of the filtrate. Since the first addition of 0.02 per cent cephalin to the blood filtrate increased the color value by 16.5 per cent (128.2-111.7 per cent) of the standard, 0.0182 per cent cephalin should produce an increase of about 15 per cent of the standard. Since an increase of 15 per cent of the color of the 0.02 per cent mixed salts means 16.5 mgm. bile salts per 100 cc. $(0.02 \text{ X} \frac{5.5 \text{ X } 15}{100} \text{ g})$, the normal cephalin content would contribute 16.5 mgm. toward the color of "blood salts."

TABLE VI.

The color value of cephalin when added to the filtrate (5 cc. A : A ; blood salts 111.7% of the standard). Standard : 0.02% mixed salt.

Conc. of Cephalin %	Found calculated as % Standard	Increase per 0.02% Cephalin
0.02	128.2	
		28.6
0.04	156.8	
		22.5
0.06	179.3	
		4.7
0.08	184.	
		5.0
0.10	189.	
		3.0
0.12	192.	

As with the lecithin experiments, addition of higher concentrations of cephalin results in a lessening of the color value increase probably due to scum formation.

(3) Cephalin and bile salts.

The color value of various concentrations of cephalin containing 0.02 per cent bile salt is shown in Table VII.

TABLE VII.

The color value of bile salts plus varying conc. of cephalin. (0.02% bile salt as a standard).

Conc. of Cephalin %	Found calculated as % Standard	Increase per 0.02% Cephalin
0.02	88.8	
		35.6
0.04	124.4	
		45.0
0.06	169.4	
		17.2
0.08	186.6	
		5.9
0.10	192.5	
0.12	?	
		7.3
0.14	207.	

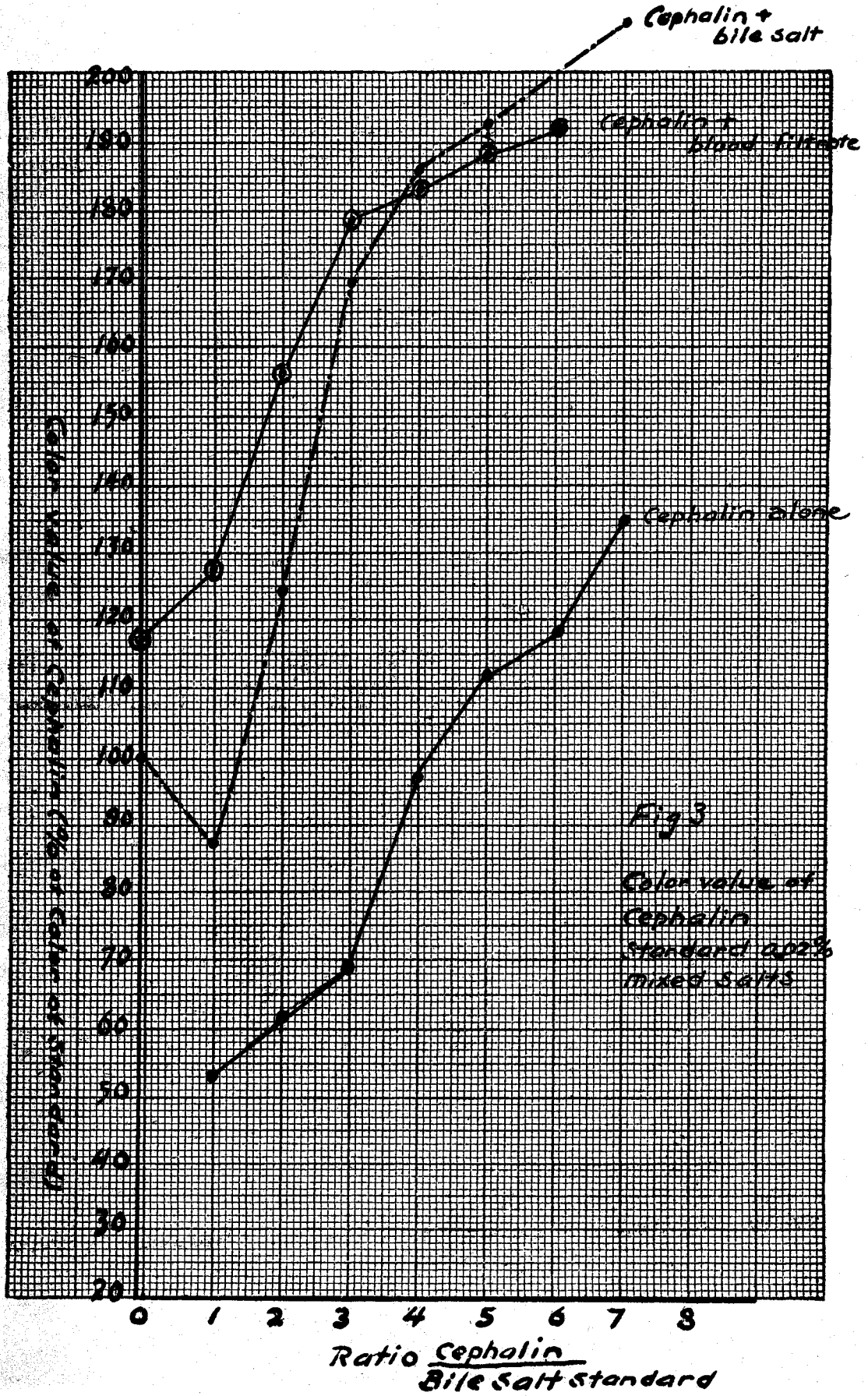


Fig 3

Color value of Cephalin standard and mixed salts

As with lecithin, the first 0.02 per cent addition of cephalin to 0.02 per cent bile salts gives about 10 per cent loss in color, but the next addition causes an increase of about four times as much as that of cephalin alone. It is a remarkable fact that addition of the bile salts should change the rate of the color increase of both cephalin and lecithin. All the data obtained with cephalin are given in Figure 3, on page 68.

As Figure 3 shows, the curve of cephalin-bile salt does not bend as suddenly as that of cephalin-filtrate. In this connection, it is interesting to add that the mixture of cephalin and bile salt does not produce scums contrary to what we found with lecithin and bile salt.

(*D*) *The color value of cholesteryl oleate.*

(1) Cholesteryl oleate alone.

The results obtained with cholesteryl oleate alone are given in Table VIII.

TABLE VIII.

The color value of varying concentrations of cholesteryl oleate. (0.02% *bile salt as standard*).

Conc. Chol. Oleate %	Found calculated as % Standard	Increase per 0.02% Chol. Oleate
0.02	43.3	
		4.9
0.04	48.2	
		10.1
0.08	68.5	
		8.0
0.16	100.0	
		11.9
0.24	147.8	

The results show that cholesteryl oleate has about the same color producing value as lecithin when compared on the basis of equal weight. A comparison of Figure 2 and Figure 4 will show that these two curves are practically identical, showing that not only are their color values identical, but also their increases in color per increment are the same.

(2) Cholesteryl oleate and blood filtrate.

The effect of addition of cholesteryl oleate to the blood filtrate is the opposite of the effect of similar addition of lecithin and cephalin. It will be recalled that the addition of lecithin or cephalin to blood filtrate (or bile salt, too) produces more color per 0.02 per cent increase than do these compounds alone. Addition of cholesteryl oleate in the same manner has just the opposite effect. An addition of 0.24 per cent cholesteryl oleate to blood filtrate produces actually less color than that of the ester alone. Not only is the color less than the sum of the theoretical, but also the increase per 0.02 per cent increment is less for practically all concentrations of the oleate. This must undoubtedly be due to a loss during hot acetone treatment.*

TABLE IX.

The color value of filtrate + varying concentrations of cholesteryl oleate. Filtrate equals 73.6% standard.

Conc. Chol. Oleate %	Found calculated as % Standard	Increase per 0.02% Chol. Oleate
0.02	79.8	
		4.8
0.04	84.6	
		1.5
0.08	87.5	
		3.9
0.16	113.0	
		3.7
0.24	127.8	

The normal cholesteryl oleate content of human blood is not known. The amounts of total cholesteryl esters found in the plasma are variable. Generally they amount to about 100 mgm. per 100 cc. of blood. It is very doubtful that all of this could be the oleate, but merely for the sake of convenience, let us assume so. Assuming that all the oleate goes into our filtrate and that none is lost during the hot acetone treatment, we should find 0.0182 per cent cholesteryl oleate in the final solution. Now an addition of 0.02 per cent of the oleate in the final filtrate increased 6.2 per cent of the color of the standard. Thus 100 mgm. cholesteryl oleate cannot contribute more than the color equivalent to 6.8 mg. bile salts, and actually far less.

(3) Cholesteryl oleate and bile salts.

The results of analyses of this combination are given in Table X.

TABLE X.

The color value of 0.02% *bile salt plus varying concentrations of cholesteryl oleate.* (0.02% *bile salt as standard*).

Conc. Chol. Oleate %	Found calculated as % Standard	Increase per 0.02% Chol. Oleate
0.02	107.7	
		9.6
0.04	117.3	
		4.5
0.08	126.3	
		6.
0.16	150.4	
		4.2
0.24	167.5	

*As matter of fact according to Page and Rudy (9), the solubility of cholesteryl oleate in alcohol is 0.3 per cent, and in acetone 2.4 per cent at 20°. Since the solubility of the oleate in hot acetone is 7.2 per cent, we must lose it in a considerable amount during acetone treatment for removal of cholesterol. A partial saponification would probably change these, as Walker pointed out (2).

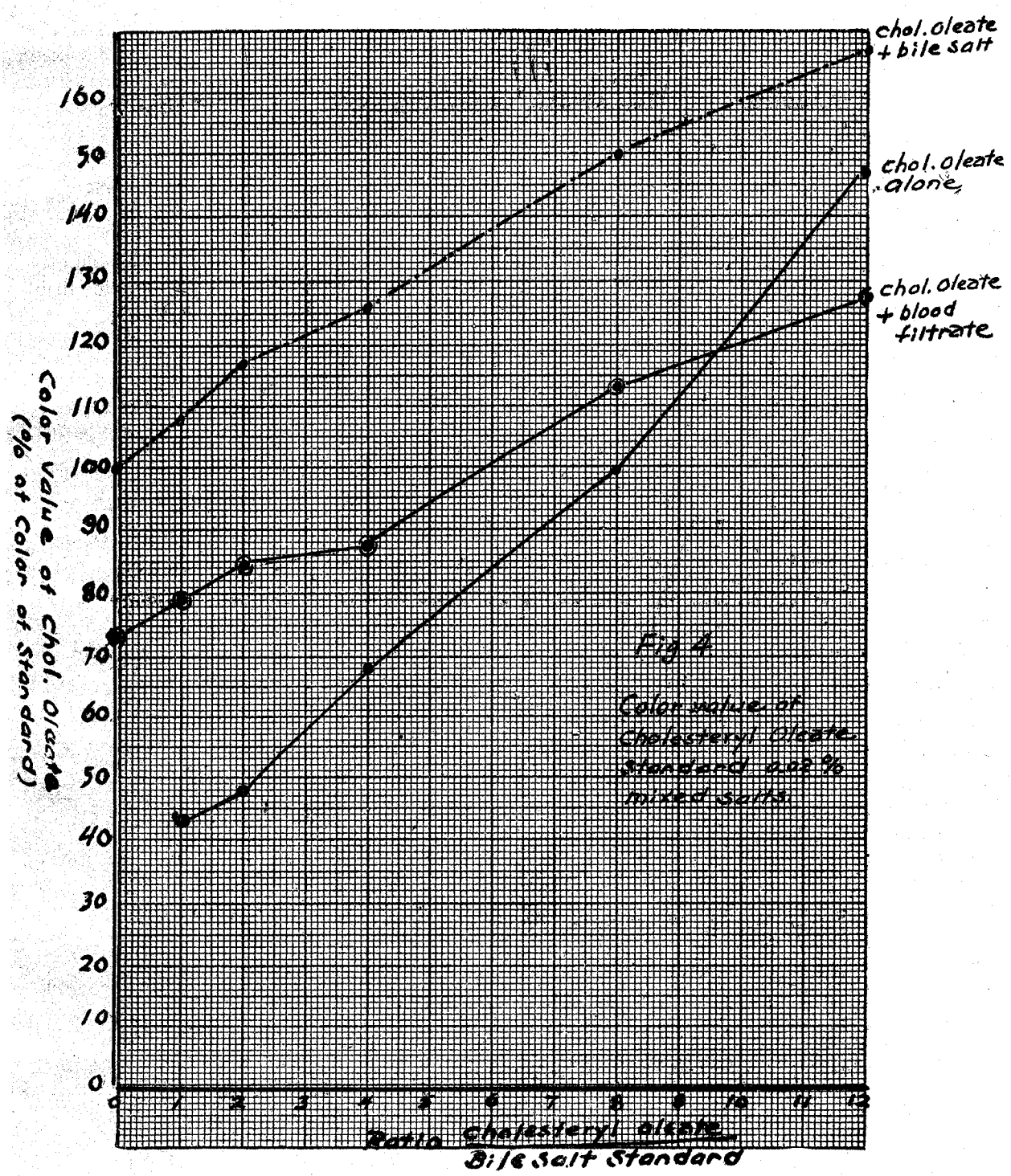


Fig. 4.

Color value of Cholesteryl Oleate. Standard 0.02% mixed salts.

One outstanding difference between these results and those obtained with the similar mixture of lecithin and cephalin is that the first addition of 0.02 per cent oleate to 0.02 per cent bile salts, gave a theoretical increase, in contrast to the others when the color values were less than that of the bile salt alone. The color values of cholesteryl oleate under these three conditions are illustrated in Figure 4, on page 70.

DISCUSSION

A review of the results obtained would indicate that about 60 mgm. per cent of the blood salts is due to these lipids. This is indeed a great deal, but it still leaves for normal human blood over 70 mgm. "blood salts" unaccounted for. It is quite possible that this remainder may yet be lessened when other Pettenkofer positive substances are investigated.

But as far as these lipids are concerned, 60 mgm. per cent probably represents the maximum for normal blood, and the greater part of the color of blood salts cannot be explained by these lipids. As will be shown later (10), in the majority of pathological bloods as well as in the blood of experimental animals and normal humans, an increased "blood salts" is accompanied by a decrease of phospholipids, which alone should contribute a little over 40 mgm. per cent of "blood salts," and the contribution by cholesteryl oleate is a little over 6 mgm. per cent.

It appears from this that the greater part of the variation of "blood salts" in normal and pathological cases must be due to something else. Could it be due to deoxycholic acid?

In conclusion we should add one reserving clause here. We have indicated repeatedly that the presence of the blood constituents modifies the Pettenkofer color of these lipids as well as that of the different bile salts. It is quite possible that an addition of 200 mgm. of lecithin, 100 mgm. of cephalin, and 100 mgm. of cholesteryl oleate all together to 100 cc. of normal blood may produce a color value, entirely different from that equivalent to 60.3 mgm. per cent of the mixed salts, if there is a mutual influence among them. An investigation of this possibility is now under way. (See Addendum, page 73.)

ULTRAVIOLET FLUORESCENCE

In Studies I, one of us reported that the ultraviolet fluorescence of the mixed salts and the blood salts is quite different, that of the mixed salts being reddish purple, and that of the blood salts being purplish blue. And these fluorescences can be easily identified by comparing them to Clark's Indicator chart placed under the same filter of the ultraviolet ray described before. The results of further studies of the different bile salts and the bloods are given in Table II.

In these experiments the different solutions were treated as though one were determining their color value, that is, it is the fluorescence of the Pettenkofer color as prepared by Tashiro's method.

TABLE XI.

Fluorescence as excited by ultra-violet rays, filtered through purple Corex filter. (Almost monochromatic light at line 3660 A. U. with about 90% efficiency).

Substance from which Pettenkofer Color is Prepared	Fluorescence Color	Equivalent to Fluorescence of the Pigment used in Indicator Chart of Clark
The Mixed Salt	Reddish Purple	Bromo Phenol Blue pH. 4.6.
Free Cholic Acid	Reddish Purple	Bromo Phenol Blue pH.4.2–4.4
Free Deoxycholic Acid	Purplish Blue	Thymol Blue. pH.9.6–9.8
"Blood Salts"	Purplish Blue	Thymol Blue pH.9.6–9.8

The difference in ultraviolet fluorescence is so striking that it gives a very easy method of distinguishing cholic acid from deoxycholic acid. Since the spectrum analysis of these fluorescent colors is not yet complete, it is difficult to say whether or not fluorescent colors of deoxycholic acid and the blood salts are absolutely identical. But the fact that these two are so alike to the naked eye, and so totally different from the mixed salts and cholic acid, is very significant.

THE MINIMUM AMOUNT OF THE MIXED SALTS DETECTABLE WITH ULTRAVIOLET FLOURESCENCE.

Since we have not yet found any cholic acid color in all the blood we examined, except in one case (see Studies I), it was interesting to deter-

mine how small amounts of cholic acid we could detect with this method.

In this experiment, we took the filtrate of horse plasma (6 H PO), which contained 0.0856 per cent "bood salts," and added to it various amounts of the mixed salts. In the control, we took the same amount of the mixed salts without the filtrate. It was the purpose of these experiments to determine the minimum concentration of the cholic acid group we could detect, and see if this minimum concentration is altered when the filtrate is present. It is reasonable to expect that if the filtrate contained some cholic acid, a less amount of the mixed salt need be added in order to give the fluorescence. The results are given in Table XII.

TABLE XII.

The minimum cholic acid detectable with ultra-violet fluorescence.

cc. Blood Filtrate	cc. 0.05% Mixed Salt Added	Final Concen. of added Mixed Salts*	Cholic Acid fluores-cence
0	0.05	0.001%	—
0	0.1	0.002%	—
0	0.5	0.01%	+
0	1.	0.02%	+
5 cc.	0.05	0.001%	—
5	0.1	0.002%	—
5	0.5	0.01%	+
5	1.	0.02%	+

* In all cases, the mixture was evaporated and dissolved in 2.5 cc. alkali, and 1 cc. was used for each determination.

Although the increment of bile salts added is not small enough to give the exact border line, a 0.01 per cent solution can surely be detectable by this method. So far as these experiements go, the prescence of blood filtrate does not alter this minimum point. As a matter of fact, even with 100 cc. filtrate, this sample did not give the characteristic fluorescence without the addition of the mixed salt, showing that it could not have more than 0.0027 per cent cholic acid group, if it has any at all.

We may add here the result of a very preliminary experiment with the blood of the portal vein. We fed 0.2g of mixed salts to a rabbit at 11 A. M., and took the sample of the blood from heart and portal vein at 2 P. M. The latter sample contained 0.1980 per cent "blood salts," and the former 0.1772 per cent. For some reason this rabbit showed a high "blood salts" figure. At any event, we could not detect any cholic acid fluorescence in the Pettenkofer color prepared from both samples.

The question whether the amount of cholic acid group present in the portal vein under the conditions is too small to be detected by fluorescence or due to a change of cholic acid to other acid such as deoxycholic acid during the passage of the intestine, must be decided by further experiments.

CONCLUSION

(1) Pettenkofer reaction of three lipids were tested by our quantitative method. Lecithin behaves in some respect like deoxycholic acid and "blood salts." Its Pettenkofer color becomes intensified when added to blood filtrate. The same thing is true of cephalin, but cholesteryl oleate behaves in the opposite way.

(2) The combined color value of these three cannot be greater than that equivalent to 60 mgm. per cent of bile salts.

(3) Blood filtrate contains something which has the power of intensifying the Pettenkofer color of lecithin. Bile salts have a similar power.

(4) Fluorescence of the pigments from the "blood salts" and from deoxycholic acid are very much alike. Fluorescent colors of "blood salts" and lecithin are similar, although that of the latter is usually more yellowish than that of "blood salts."

(5) Cholic acid can be easily distinguished from deoxycholic acid by this characteristic ultra-violet fluorescence.

(6) The mixed salt can be detected in 0.01 per cent solution by this method. If 5 cc. of a filtrate give the reaction, the original sample must contain more than 0.055 per cent cholic acid group; and if 10 cc. of a filtrate give it, the original must have more than 0.0275 per cent. Of the pathological samples, we found only one case where we were able to demonstrate its presence with 10 cc. or 5 cc. filtrate.

(7) Normal horse blood cannot have more than 0.0027 per cent cholic acid, if it contains any at all.

(8) The fact that we could not detect cholic acid in the portal vein of the rabbit after ingestion of bile salts in a single experiment, will bear further investigation.

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ADDENDUM

As a matter of fact, the results of preliminary experiments show that the combination of these three compounds give about half of the sum of the color found with each. Considering the blood to contain 220 mg. lecithin, 110 mg. cephalin and 110 mg. cholesteryl oleate, the final concentration would correspond to 0.04 per cent lecithin, 0.02 per cent cephalin and 0.02 per cent cholesteryl oleate, since in our method, 1 cc. of the final extract corresponds to 1/5.5 cc. of blood. Thus: (1) When we take a solution containing 0.04 per cent lecithin, 0.02 per cent cephalin and 0.02 per cent cholesteryl oleate and compare it with a 0.02 per cent bile salt standard, we find that this mixture gives a color value of 75 per cent of that of the standard. If one considers the theoretical sums of the color values of these substances at these concentrations determined separately, they would represent 148 per cent of the standard. (2) If we add this same mixture to the standard bile salt, the color of the standard is increased only 35 per cent. This 35 per cent increase is less than half of that which we would expect to find. (3) If we add this mixture to a blood filtrate having an original color value of 93.5 per cent of the standard, we obtain an increase of 38.4 per cent—again only half of the expected increase.

These results show that a mixture of the above-named lipoidal substances give a color less than the sum of the color values of the substance alone. When this mixture is added to a blood filtrate or to bile salt, the color value change produced by addition of the mixture is much smaller than the theoretical value. It is therefore obvious that these substances will contribute not more than 40 mgm. of the "blood salts" color, instead of the 60 mgm., as stated above—leaving an even larger value for Pettenkofer positive substance unaccounted for in our "blood salts" determination.