MECHANISM OF THE REACTION BETWEEN BILE SALTS AND BLOOD SERUM AND THE EFFECT OF CONJUGATION IN THE FORMATION OF BILE SALTS.[1]

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The antagonistic action of blood serum and bile is best shown by the use of red blood corpuscles. In a suitable mixture of a hemolytic serum and bile salts no hemolysis occurs (1). The conditions of the experiment might be arranged so that either the serum alone or the bile salts alone would cause active hemolysis, but each of the two hemolytic agents when mixed in the proper proportions destroys the action of the other and they afford a good medium for the preservation of the red cells. A similar phenomena is shown, less strikingly, by substituting typhoid bacteria for the red corpuscles, for example, in the cultivation of *B. typhosus* from the blood by the use of bile media.

Thus far no property of the bile salts has been investigated which is not influenced by serum and this relationship must be taken into consideration in studying the biological action of these salts. It has been found that serum inhibits the poisonous action of bile on the blood corpuscles, on muscle, on the vascular and central nervous system (1), and also delays the action of a lethal dose of bile salts (2).

It is possible to consider either that the serum possesses separate inhibiting properties for each of the tissues tested or that a single reaction between the bile salts and serum destroys a certain group of properties. The latter explanation is the simpler and is preferable, unless further developments make it necessary to accept a more complicated one. The bacteria or blood corpuscles, or whatever tissue

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is employed, serves merely as an indicator to show whether or not a reaction has occurred.

The physiological importance of this protective action of the serum is evident. The interest associated with it depends in part, however, upon the specificity of the reaction, *i. e.*, whether it is a general property of all tissues, both plant and animal, or whether it is peculiar to the blood. One may ask whether it is merely an accidental occurrence or whether, to meet an emergency, the organism has developed a special adaptation. An analysis of the mechanism of the reaction has some bearing on this question and may be outlined as follows: (1) What is the active part of the bile salt molecule and what is the active constituent of the serum? (2) Is the property of inhibition peculiar to serum or is it common to both plant and animal tissues? (3) Does the synthesis of cholalic acid with glycocoll have any effect on its hemolytic reactions? (4) Does serum inhibit hemolytic agents to whose action the body has not been exposed?

Active Part of the Bile Salt Molecule.—Sodium glycocholate, when hydrolyzed, splits into glycocoll and cholalic acid. In testing the hemolytic power of these two parts,[2] glycocoll was found to have no solvent action in concentrations varying from 0.001 to 1 per cent. Cholalic acid is only very sparingly soluble, one part dissolving in 4,000 parts of water. But even at this dilution a saturated solution in physiological salt solution gave complete hemolysis.

[2] A uniform technique has been employed throughout the various experiments. Any variations which were necessary in the following description have been noted in the individual experiments. Physiological sodium chloride (0.85 per cent.) has been used as a solvent and diluting agent for all material, whatever, entering into the preparations. Serum and corpuscles were obtained by defibrinating human blood. The serum was separated by centrifugalization and the sediment of corpuscles was washed three times, using about 0.5 c.c. of corpuscles to 10 or 12 c.c. of physiological salt solution. The sediment of corpuscles was diluted to a 2 per cent. emulsion. In comparing the action of the various chemical agents, equi-molecular solutions were used in every instance. The solutions were prepared by direct weighing without any further standardization, and progressive dilutions were made from these stock solutions. The concentrations given in the tables represent the original strength of the solution before dilution by the addition of corpuscles. The final preparations consisted of 1 c.c. of the hemolytic agent and 0.5 c.c. of the emulsion of corpuscles, the final volume being 1.5 c.c. This volume was

In order to obtain a comparison of the hemolytic activity of glycocholic and cholalic acids, the sodium salts were used on account of the insolubility of the free acids. The result, given later in Table III, shows that the cholalate group is the essential factor in the production of hemolysis. Also its hemolytic activity is at most only slightly greater than corresponding quantities of sodium glycocholate. The cholalic acid radical is the part of the molecule which is generally active, being responsible for the Pettenkoffer color test, for the acceleration of ferments (5) and for bactericidal action (6).

Active Constituent of the Serum.—The evidence thus far available is almost conclusive that it is the proteid portion of the serum which inhibits hemolysis by bile salts (3, 4). Deleterious agents, such as heat,[3] exert little or no effect on the inhibiting property. Thus a 1 to 10 dilution of serum which has been heated to 95° or 100° C. is as active as an unheated serum. It is important, however, to note that on heating diluted serum only a slight cloud forms and most of the proteids remain in solution as alkali albumin. The following experiment indicates that the proteids possess active inhibitory properties.

One part of normal rabbit serum was mixed with four parts of absolute ethyl alcohol and the precipitate filtered off. The precipitate was dried cautiously on the water bath and the filtrate evaporated to dryness, also on a water bath. The two portions were then made up to their original concentrations and tested in 1 to 10 dilution. The fraction obtained by precipitation gave well marked inhibition of hemolysis, even better than the same dilution of the corresponding serum whereas the fraction from the filtrate did not differ from salt solution. The data include also the effect of heating the serum in a 1 to 10 dilution.

not increased where serum or other inhibiting agents were added. The serum was used in 1 to 10 strength, the proper quantity being added directly to the emulsion of corpuscles to give the desired dilution. The further dilution which resulted upon the addition of 1 c.c. of the hemolysin made a final dilution of 1 to 30 for the serum. The preparations were incubated for two hours at 37° C. and then left over night at about 8° C.

[3] *Bull. of the Johns Hopkins Hosp.*, 1908, xix, 268.

TABLE I.

Effect of the Proteids of Serum on Hemolysis.

Sodium glycocholate per tube.	Normal rabbit's serum.	Rabbit's serum 1–10 heated 20 minutes at 90° C.	Salt solution.	Precipitated proteids.	Filtrate salts and extractives.
3 mg.	C	C	C	C	C
2 "	C	C	C	C	C
1 "	Tr	Tr	C	Tr	C
0.8 "	Tr	Tr	C	O	C
0.5 "	Tr	Tr	P	O	P
0.2 "	O	O	Tr	O	Tr
0.09 "	O	O	O	O	O
salt solution	O	O	O	O	O

C represents complete hemolysis; P, partial hemolysis; Tr, trace of hemolysis; O, no hemolysis.

These results would prove that the proteid and not the salts and extractives are the important constituent of the serum, except for the possibility that some other substance was mechanically carried down in the precipitate.

Specificity of the Reaction.—The reaction is apparently highly specific and is satisfactorily given only by proteids obtained from the serum. Meyer (4) found that not only do plant proteids, such as edestin, exert no inhibitory action, but even animal proteids, such as egg albumen, also possess no inhibitory power.[4] The following results were obtained with fresh egg albumen and with the egg and blood albumin which is put on the market by Kahlbaum. The white of egg was used in 1 to 10 dilution in salt solution and for the albumins, 0.5 gram was dissolved in 100 c.c. of physiological salt solution, a concentration of proteid which roughly approximates a 1 to 10 dilution of serum.

In the following table the inhibiting power shown by the egg albumen is practically negligible.

Effect of the Synthesis of Glycocoll with Cholalic Acid.—The animal organism utilizes a series of chemical methods by which a variety of poisons may be rendered partly or entirely harmless. A classical example of such a method is the formation of a conjugation

[4] Donati and Satta (7) have recently reported results which apparently contradict these conclusions. Complete inhibition of hemolysis by bile salts was obtained with 5 per cent. white of egg solution and by a 10 per cent. solution of edestin.

TABLE II.

Relative Inhibiting Power of Serum and of Egg Albumen.

Sodium glycocholate per tube.	Control salt solution.	Normal pigeon serum.	White of egg.	Albumin, 0.5 gr. per 100, from	
				Blood.	Eggs.
2.0 gm.	C	C	C	C	C
1.0 "	C	O	C	C	C
.8 "	C	O	C	C	C
.5 "	C	O	C	P	C
.2 "	C	O	C	O	C
.1 "	P	O	O	O	P
.09 "	Tr	O	O	O	O
Salt solution.	O	O	O	O	O

C represents complete hemolysis; P, partial hemolysis; Tr, trace of hemolysis; O, no hemolysis.

product such as the synthesis of phenol with sulphuric or glucuronic acids, and of benzoic acid with glycocoll to form hippuric acid. Of the conjugation products which are normally present in the body, the bile salts furnish one of the most prominent examples, the cholalic acid being combined usually with glycocoll or taurine. Their reaction with glycocoll is represented by this equation:

$$C_{23}H_{39}O_3COOH + H{\cdot}NH{\cdot}CH_2COOH = C_{23}H_{39}O_3CONH_2COOH + H_2O.$$

It is quite conceivable that this conjugation might affect the physiological properties of cholalic acid just as other poisons are modified by conjugation. It is of some interest that although a great number of bile salts have been studied and described (8) such as the glycocholate, taurocholate, glycocholeate, cheno-taurocholate, and scymnol sulphate, yet apparently no observer has found the free cholalate nor the related chololeate and fellate occurring unconjugated in the bile.

It may be determined directly by animal inoculations in the case of phenol and benzoic acids, that the conjugation has, to a considerable extent diminished the toxicity of the original compounds. Relatively small doses of the original poisons will produce death as compared with the large amounts of the conjugated products which the animals can withstand. In the case of the bile salts, however, the sodium glycocholate is highly poisonous and we were not able

to show that the lethal dose of sodium cholalate was definitely smaller than that of the sodium glycocholate. By intraperitoneal inoculations into guinea-pigs weighing from 230 to 250 grams, 0.1 gram of sodium glycocholate was found to be fatal in from ten to eighteen hours. The injection of 0.09 gram of sodium cholalate intraperitoneally into a guinea-pig weighing 270 grams caused death after forty-eight hours only. A second guinea-pig of the same weight, receiving a duplicate injection, survived without symptoms. Although slight differences have been reported in the toxicity of sodium taurocholate and glycocholate, the lethal dose of the cholalic acid is evidently not strikingly affected by the conjugation. Only minor differences in symptoms were observed in the two sets of guinea-pigs, and apparently animal inoculations do not afford a very suitable method for comparing the two substances.

TABLE III.

Hemolytic Activity of the Different Bile Salts and the Relative Inhibiting Power of Serum for each.

Strength of solutions in fractions of normal.	Approximate weight[6] of bile-salt in mgs. per tube.	Sodium taurocholate.		Sodium glycocholate.		Sodium cholalate.	
		Salt solution.	Serum 1–10.	Salt solution.	Serum 1–10.	Salt solution.	Serum 1–10.
1/250	2	C	C	C	C	C	C
1/500	1	C	O	C	O	C	C
1/625	0.8	C	O	C	O	C	C
1/833	0.6	C	O	C	O	C	C
1/1,250	0.4	C	O	C	O	C	C
1/2,500	0.2	C	O	C	O	C	O
1/5,000	0.1	C	O	C	O	C	O
1/5,555	0.09	C	O	C	O	C	O
1/6,250	0.08	P	O	C	O	C	O
1/8,333	0.06	Tr	X	C	X	C	X
1/12,500	0.04	Tr	X	Tr	X	C	X
1/25,000	0.02	O	X	O	X	O	X
1/50,000	0.01	O	X	O	X	O	X
Control salt solution.		O	O	O	O	O	O

C represents complete hemolysis; P, partial hemolysis; Tr, trace of hemolysis; O, no hemolysis.

One might expect that on injecting the cholalic acid conjugation could occur so rapidly that it would be practically equivalent to

[6] Although the corresponding weights of the three salts are all necessarily different, yet this column represents at least the order of magnitude of the weight of any one in a given tube.

injecting the glycocholate. Such complications can be partly avoided by employing the test tube reactions which have been developed in connection with the hemolytic action of bile salts. In the following experiment a comparative test was made of the hemolytic activity of sodium taurocholate, glycocholate and cholalate, together with the inhibiting action of serum on these substances.

Two successive repetitions of this experiment gave results similar to those in the preceding table, except that as the solutions grew older they became somewhat less actively hemolytic. Some of the stronger solutions of the sodium cholalate precipitated on standing, but this did not occur in the more dilute solutions and apparently did not interfere in any way with the reactions.

In another set of experiments, preliminary digestions were arranged which were designed to give the serum the most favorable opportunity for inhibiting the action of the cholalate. Mixtures were prepared similar to those in Table III, and in addition two series of tubes were added. In one, the corpuscles were digested with the serum, and in the other, the cholalate was digested with the serum before the corpuscles were exposed to the hemolytic agent. The data confirmed those in Table III. In the corresponding preparations containing serum the results were all practically the same, regardless of the preliminary digestion. An analogous experiment with sodium glycocholate also gave the conclusion that the preliminary digestion did not alter the final result.

TABLE IV.

Hemolytic Action of Cholalic Acid and the Effect of Serum.

Concentration of cholalic acid in percentage of saturation.	20 per cent. emulsion.		5 per cent. emulsion.	
	Salt solution.	Serum 1–10.	Salt solution.	Serum 1–10.
100	C	P	C	C
75	Tr.	O	C	C
50	O	O	C	O
25	O	O	O	O
Salt solution.	O	O	O	O

C represents complete hemolysis; P, partial hemolysis; Tr, trace of hemolysis; O, no hemolysis.

The results of the experiments with free cholalic acid are given in Table IV. Progressive dilutions of a saturated solution of the rather insoluble acid were tested, and to increase the hemolysis one series of tubes was prepared with a very small amount of corpuscles. In order to avoid further dilution of the cholalic acid, instead of the usual volume of 0.5 cubic centimeter of corpuscles, a smaller volume, namely, 0.05 cubic centimeter of a twenty per cent. and of a five per cent. emulsion was employed.

A review of Table III shows that for the production of complete hemolysis in the presence of serum the amount of glycocholate required is thirty-three times greater than in the corresponding serum-free preparations; of the taurocholate, twenty-two times greater; and of the cholalate, ten times greater. Slightly less pronounced results were obtained where some of the older preparations of bile salt were used. The amount of glycocholate or taurocholate, however, required to produce complete hemolysis in the presence of a 1 to 30 dilution of serum was always at least twice as great as the amount of cholalate required for the same result. The data obtained with the free acid confirm the general conclusions derived from the tests with the sodium salt. Although the serum possesses some inhibiting power against the cholalate, the results have consistently shown that the inhibition is measurably less than for the glycocholate and taurocholate. The protective mechanism may be considered as consisting of two factors: first, the conjugation of the cholalic acid and, secondly, the inhibition of the still toxic conjugation product by means of serum.[6] These experiments do not offer any explanation as to why the serum inhibits the conjugated salts more effectively than the unconjugated.

Foreign Toxins.—Several foreign hemolytic agents were tested,

[6] Rywosch (9) came to the conclusion that the toxicity of cholalic acid was increased by conjugation with taurine and diminished by conjugation with glycocoll. Frogs, injected subcutaneously, were killed by 0.06 to 0.07 gram of the taurocholate, by 0.08 gram of the cholalate, and by 0.1 gram of the glycocholate. Similar conclusions were obtained from the hemolytic experiments. The serum and corpuscles were not separated, but blood was diluted 10 to 20 times with physiological salt solution. Sodium taurocholate caused hemolysis in dilutions of 1 to 600; the cholalate, in 1 to 200; and the glycocholate, in 1 to 50 dilution. We were not able to confirm these differences in hemolytic activity, either with washed corpuscles or in mixtures containing serum.

namely, tetanolysin, phenol, sodium benzoate, and ethyl alcohol. In 1 to 30 dilution normal serum showed at most only a minimal inhibiting power against these agents. In the case of phenol and tetanolysin only a trace of hemolysis was restrained. With sodium benzoate and alcohol the serum was never able to inhibit as much as twice the minimal dose required for complete hemolysis. On the other hand, with the bile salts the serum readily inhibited as much as ten times the minimal amount required for complete hemolysis. Korschun and Morgenroth (10) found that normal serum possessed a very definite restraining action of the hemolysis caused by pancreatic extracts. This agent may be classed with the bile salts as an hemolysin to which the organism may occasionally be exposed.

General Method.—The hemolytic experiments furnish a fairly general method for studying the various reactions which occur in the body. The conjugations are of interest in connection with the results which were obtained for the bile salts. Two other conjugations were studied, namely, those occurring in the production of hippuric acid and of phenylsulphuric acid. The conclusions in regard to sodium hippurate were that the conjugation had destroyed the hemolytic power of the original sodium benzoate. In concentrations of three-fourths normal, sodium benzoate solutions produced complete hemolysis, whereas sodium hippurate solutions in double normal strength caused no lysis. The hippurate solutions changed the color of the corpuscles to a dark brown, and this alteration of the pigment did not occur in the mixtures prepared with sodium benzoate. Normal serum in 1 to 30 dilution exhibited no restraining effect against the lysis caused by the benzoate or the discoloration from the hippuric solutions.

For comparison with phenol, the potassium salt of the hypothetical phenylsulphuric acid ($C_6H_5OSO_2OK$) was prepared according to Baumann's (11) method from phenol, potassium hydroxide and potassium pyrosulphate. The crude product was purified by four recrystallizations from 95 per cent. alcohol. During the preparation proper precautions were taken to prevent the rearrangement of the potassium phenyl sulphate to form its isomere potassium-sulphophenol, $C_6H_4OH(1)SO_3K(4)$, and as a control this isomere was included in the hemolytic tests. Since it is necessary to use a salt

of phenylsulphuric acid, the potassium salt of phenol was also tested.

That these four substances were all distinct chemically is shown by the fact that they all differed in their behavior toward ferric chloride. The phenol and the potassium sulpho-phenol both gave color reactions, the sulpho-derivative being of a rather red color as compared with the phenol. The potassium phenylate gave a yellowish brown precipitate and the potassium phenylsulphate gave no reaction. As seen from Table V, they also differed in their hemolytic actions.

TABLE V.

Hemolysis by Phenol and its Derivatives.

Strength of solution in terms of normal.[7]	Phenol.		Potassium phenylate.		Potassium phenyl-sulphate.		Potassium p-sulpho-phenol.	
	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.	Serum.
1/2	Coagulated	Coagulated	C	C	C	P	O	O
1/4	Coagulated	Coagulated	C	C	O	O	O	O
1/10	C	C	C	C	O	O	O	O
1/15	C	C	C	C	O	O	O	O
1/20	C	Tr.	C	C	O	O	O	O
1/30	P	O	C	C	O	O	O	O
1/50	O	O	C	C	O	O	O	O
Control	O	O	O	O	O	O	O	O

C represents complete hemolysis; P, partial hemolysis; Tr, trace of hemolysis; O, no hemolysis.

This table presents the following points of interest:

1. The addition of serum in 1 to 10 dilution has practically no effect on any of the four substances tested.

2. The hemolytic activity of phenol on red corpuscles is definitely diminished by its conjugation. In the weaker solutions phenol causes hemolysis, but in the stronger solutions lysis is prevented by the fixation and coagulation of the cells. On the other hand, the potassium phenyl-sulphate caused no coagulation in the concentrations tested and the amount required for complete hemolysis was ten times greater than for phenol.

3. The control preparations with potassium phenylate show that

[7] Expressed in percentages, the ½N. solution for phenol would be 47 per cent.; for potassium phenylate, 6.6 per cent.; and for the two isomeres, 10.6 per cent.

the substitution of the hydrogen of the hydroxyl group by potassium not only does not diminish the toxicity, but actually increases it. Additional preparations to those given in the table showed that a solution $\frac{1}{500}$ normal was sufficient to cause complete hemolysis. No coagulation of the cells was observed, but in addition to the hemolysis the pigment turned green in all the preparations except in those containing only a slight excess of the minimum required for complete hemolysis.

4. The potassium sulphophenol gave no evidence of any toxic action. This applied not only to red corpuscles, but also to fungæ which grew freely in a solution of one-half normal strength. Although this compound is still a phenol, the introduction of a new group in the para-position has produced a marked change in its properties. In this instance the chemical alteration which resulted in a diminution of toxicity for the body was also accompanied by a diminution in antiseptic properties.

SUMMARY.

These experiments suggest the following conclusions concerning hemolytic action:

1. It is probably the proteid part of the serum which inhibits the bile salts.

2. The cholalic acid group is the active part of the bile salt molecule.

3. The protection afforded by bile salts against serum is of especial interest from the following considerations: (*a*) The protective action is a property apparently peculiar to proteids obtained from blood serum. It is not given satisfactorily by egg albumen. (*b*) The conjugation of cholalic acid with glycocoll in the formation of the bile salts is of some advantage to the organism. Although the toxicity of the cholalate for red corpuscles, when free from serum, is at most only slightly diminished by conjugation, yet the blood serum possesses a greater inhibiting action for the resulting glycocholate than for the original cholalate.

4. As compared with its inhibition of sodium glycocholate, normal serum possesses relatively little inhibiting action against certain foreign hemolytic agents, such as tetanus toxin, sodium benzoate, phenol and ethyl alcohol.

5. Hemolytic experiments afford a fairly general method for studying, *in vitro,* certain syntheses occurring in the body. They avoid, largely, the complications, such as rapid chemical alteration, which might occur in animal experimentation.

Contrary to the results obtained with bile salts, the conjugation of benzoic acid and of phenol results in an effective reduction of their hemolytic action independently of the presence or absence of serum.

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