

THE ANTI-BACTERICIDAL ACTION OF THE BILE SALTS.

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BILE and the bile salts are substances of great importance in connection with typhoid fever. On the one hand, they are extensively used in differential media for the isolation of *B. typhosus* from the excreta, and in media designed to cultivate the bacillus from the blood; while on the other, the survival of the organism in the gall bladder and its association with gall stones and cholecystitis indicate that bile may play an important rôle in the etiology of the disease. It would appear therefore that a study of the mode of action of bile in culture media for the isolation of *B. typhosus* might, apart from its bearing on bacteriological technique, incidentally throw light on the far more important question of typhoid fever and the production of typhoid carriers.

The action of bile and the bile salts in favouring the growth of *B. typhosus* on differential media has been most ably investigated by Dunschmann¹, but that observer did not extend his work to explaining the uses of bile in Blood-culture, beyond the fact of its "enriching" action on the growth of the typhoid bacillus, and its power of retarding the growth of certain other organisms.

Eppenstein and Korte² called attention to the anti-bactericidal action of bile, and Conradi³, recording further experiments to the same effect, pointed to this anti-bactericidal action as explaining the utility of bile in the culture of typhoid bacilli from the blood. A feature of Conradi's work was that he found bile to inhibit the bactericidal properties of serum, a point to be borne in mind, as Gildmeister, in a recent communication⁴, attributes the use of bile in typhoid blood-culture entirely to its haemolytic action, which he supposes to liberate anti-bactericidal substances from the disintegrated blood-cells.

¹ *Ann. Inst. Pasteur*, Jan. 1909, p. 29.

² *Münch. med. Wochenschr.* 1906, p. 1152.

³ *Ibid.* p. 1361.

⁴ *Arb. a. d. Kaiserl. Gesundheitsamte*, Feb. 1910.

Pies¹, referring to Conradi's work above quoted, lays stress on the high concentration of nutrient matter in the serum as an important factor in the survival of typhoid bacilli.

Experiments carried out by the author in collaboration with Captain C. C. Cumming, R.A.M.C.², led us to the opinion that, in the isolation of typhoid bacilli from the blood, the anti-bactericidal action of the bile salts was of far greater importance than any enriching quality that they might possess. The experiments about to be recorded were undertaken with a view to elucidating as far as possible this anti-bactericidal action, in the hope that some light might in this way be thrown on the survival of *B. typhosus* in "carriers," as well as on the properties of the bile salts as constituents of media for the culture of typhoid bacilli from the blood of cases.

EXP. 1. *Object.* To confirm previous work in demonstrating the anti-bactericidal action of sodium taurocholate.

An agar slope of B. T. "Rawlings" was emulsified in 10 c.c. of sterile normal salt solution.

A series of dilutions of this emulsion were prepared of the following strengths:—

1 in 1,000; 1 in 10,000; 1 in 100,000 and 1 in 1,000,000. It was desired to compare the bactericidal effect of a 1 in 8 dilution of normal blood in sterile water with that of the same strength of blood diluted with a 0.5% solution of sodium taurocholate. It was anticipated that on mixing known volumes of the blood preparations with equal volumes of successive dilutions of the typhoid emulsion, their relative bactericidal efficiency would be manifested in their power of sterilizing the bacterial dilutions in contact with them.

TABLE I.

		Dilution Series, Typhoid Emulsion				
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000	
A.	Blood, 10 c.mm.	} Findings on plates }	+	-	-	-
	Water, 60 c.mm.					
	Dilution of emulsion, 10 c.mm.					
B.	Blood, 10 c.mm.	} Findings on plates }	+	+	+	+
	Taurocholate 0.5% solution, 60 c.mm.					
	Dilution of emulsion, 10 c.mm.					

+ = growth, - = sterile.

¹ *Arch. f. Hygiene*, 1907, LXII. p. 125.

² *Journ. Royal Army Med. Corps*, June 1910.

Series A. To 10 c.mm. of each bacterial dilution were added 10 c.mm. of freshly drawn blood and 60 c.mm. of sterile water.

Series B. To 10 c.mm. of each bacterial dilution were added 10 c.mm. of freshly drawn blood and 60 c.mm. of a 0.5% solution of sodium taurocholate. The four preparations from each series were incubated at 37° C. for 20 hours, and then spread on plates. The result is shown in Table I.

It will be seen that while the mixture of blood and water was able to sterilize a 1 in 10,000 dilution of typhoid emulsion, the presence of sodium taurocholate in a similar dilution of blood annulled all bactericidal effect on even so high a dilution of bacterial emulsion as 1 in 1,000,000.

This experiment has been frequently repeated and always with a like result. It might however be urged that the survival of the bacteria in contact with sodium taurocholate was due, not to any anti-bactericidal action of the salt but rather to the enriching power claimed for it by Dunschmann. To settle this point, it was decided to work out the enrichment power, if any, of the sample of sodium taurocholate under examination, leaving the action of blood out of the question.

EXP. 2. In separate test-tubes were placed 10 c.c. of ordinary peptone and salt solution and of a 0.5% solution of sodium taurocholate in peptone and salt. To each tube was added 10 c.mm. of an emulsion of B. T. "Rawlings," and both preparations were incubated at 37° C. for three days. A "count" of each preparation was then made, with the result that the bile salt peptone water tube contained 235,000,000 bacilli in 1 c.c. while the peptone water contained 249,000,000 per 1 c.c.

It is obvious, therefore, that the sample of sodium taurocholate under examination has no marked enriching effect in three days—in fact rather the reverse.

It may then be taken as proved that sodium taurocholate is able to inhibit the bactericidal action of normal blood.

Since it is well known that the bactericidal efficiency of the blood-fluids increases during the process of clotting and is greater in the serum than in fresh blood, it next became a question whether the bile salt acted by preventing the elaboration of bactericidal substances during clotting or interfered with their activities after elaboration.

EXP. 3. A broth-culture of B. T. "Rawlings" was diluted as in Exp. 1. A sample of blood was then withdrawn by finger-puncture, portion of it, in 1 in 4 dilution, treated at once, and the remainder allowed to clot and the serum treated in the same dilution after

10 minutes, 2 hours and 6 hours respectively. All preparations were incubated for 20 hours and plated.

The result is shown in Table II.

This experiment showed that sodium taurocholate acted, not by interfering with the formation of bactericidal substances, but by inhibiting their action.

Before proceeding further in the mechanism of this inhibition, it seemed important to ascertain whether this power was shared by the glycocholate of soda also, and whether the constituents of these salts, taurin, glycin, and cholalic acid, were able equally to interfere with bactericidal action.

TABLE II.

		Dilution Series of Typhoid Broth Culture				
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000	
A.	Fresh blood, 5 c.mm. 0.5% taurocholate solution, 10 c.mm. Dilution of emulsion, 5 c.mm.	Findings on plates	+	+	+	+
B.	As above but with "10 minutes" serum		+	+	+	+
C.	As above but with "2 hours" serum		+	+	+	+
D.	As above but with "6 hours" serum		+	+	+	+

N.B.—The broth culture dilutions were kept at a temperature of 32° F. in the intervals of being used, to prevent multiplication of the bacilli.

EXP. 4. Dilutions of broth-culture of B. T. "Rawlings" were prepared as before.

Solutions containing 0.5% of each of the above substances were made in sterile water, and a mixture of one part of fresh blood in three parts of each solution was then prepared.

To 10 c.mm. of each blood-mixture was added 5 c.mm. of each dilution of the typhoid culture, and the preparations incubated and plated as before.

The result is shown in Table III.

It appears from the above that both sodium taurocholate and glycocholate possess anti-bactericidal qualities, while glycin, taurin and cholalic acid are without any such action, sterilization of the culture being active when blood is mixed with solutions of these substances.

The cholalic acid used was an old sample which had been long in the laboratory, and it will be desirable to further test this acid when a

TABLE III.

		Dilution Series of Typhoid Broth Culture					
		1 in 1,000	1 in 10,000	1 in 100,000	1 in 1,000,000		
A.	Fresh blood 1 part Sterile water 3 parts Dilution of culture, 5 c.mm.	10 c.mm.	} Findings on plates	+	-	-	-
B.	Fresh blood 1 part 0.5% sol. of taurocho- late of soda 3 parts Dilution of culture, 5 c.mm.	10 c.mm.		}	+	+	+
C.	As above, but 0.5% taurin sol.	...	„		-	-	-
D.	As above, but 0.5% glycocholate of soda sol.	}	„	+	+	+	+
E.	As above, but 0.5% glycin sol.		...	„	-	-	-
F.	As above, but 0.5% cholalic acid sol.	„	„	+	-	-	-

reliable preparation is available. It is curious that, in view of the proved absence of anti-bactericidal action in taurin and glycin, and the activity in this respect of the taurocholate and glycocholate of soda, the cholalic acid should be without this quality, but, assuming the sample used to be reliable, the above experiment certainly indicates that this is the case.

It is now time to return to the mechanism of this anti-bactericidal action of sodium taurocholate.

Regarding the disintegration of bacteria as a "complement-amboceptor" reaction, it may be assumed that bactericidal activity can be destroyed by preventing the action of amboceptor or of complement or both. The elucidation of this question is complicated by the difficulty of obtaining complement free from amboceptor, but this can to a certain extent be got over by comparing two sera of different bactericidal "titre."

EXP. 5. *Object.* To ascertain whether sodium taurocholate interferes with the sensitization of typhoid bacilli by amboceptor.

The serum of a rabbit possessing a considerable degree of immunity to *B. typhosus* and agglutinating it in a dilution of 1 in 200, was heated for 25 minutes at 60° C. to inactivate its complement. The serum of a normal rabbit was obtained at the same time and treated in the same way. A 24 hours' agar culture of B. T. "Rawlings" was emulsified in saline.

The following mixtures were then prepared :—

(1)	Heated immune serum	1 part
	0·5 % sodium taurocholate solution ...	1 part
	Typhoid emulsion	2 parts
(2)	Heated normal serum	1 part
	0·5 % sodium taurocholate solution ...	1 part
	Typhoid emulsion	2 parts
(3)	Normal salt solution	2 parts
	Typhoid emulsion	2 parts
(4)	Normal salt solution	1 part
	0·5 % sodium taurocholate solution ...	1 part
	Typhoid emulsion	2 parts

These mixtures contained, in all cases, the same concentration of typhoid emulsion, and where it was present, of sodium taurocholate. They were left in contact, over night, at room temperature to ensure sensitization of the bacteria, if this indeed could take place in the presence of the taurocholate, in the serum preparations.

The mixtures were then centrifuged for 45 minutes, the deposits washed in saline, and again centrifuged. After a final washing the deposits were emulsified in saline, the emulsions being "matched" by opacity to eliminate as far as possible fallacies arising through unequal multiplication and unequal "deposit"—the latter especially, as there was agglutination in *both* serum preparations, though of course this was more marked in the immune serum.

It was expected that if the bile salt had not interfered with the amboceptor in the heated immune serum, the bacteria in "mixture 1" would be so far sensitized as to be able to deflect complement to a greater extent than those placed in contact with normal serum. The saline emulsion was introduced as a "control" and the "bile salt—saline" mixture to make sure that the sodium taurocholate itself exerted no "sensitizing" action on the bacteria. The four emulsions were handed to Major W. L. Harrison, R.A.M.C., who very kindly undertook this part of the work. He examined them without knowing which emulsion was supposed to be the sensitized one, until the "key" was consulted after the experiment, and he reported as follows :—

"Complete deviation occurred when emulsion No. 1 was placed in contact with 1 in 50 complement, partial when No. 2 was ditto, and none when Nos. 3 and 4 were placed under the same circumstances.

"From your key, it appears that bile salt did not affect the amboceptor. Possibly there was enough amboceptor in your normal serum, in the quantity used, to sensitize your bacteria; hence the partial result with No. 2."

The fact that there was agglutination with the "normal" serum would support Major Harrison's surmise as to the possibility of the presence of amboceptors.

The experiment goes far to prove that amboceptor is not interfered with by sodium taurocholate, and the anti-bactericidal action of this salt is therefore probably exerted through an inhibition of complement.

EXP. 6. Object. To ascertain whether sodium taurocholate interferes with the action of complement.

The serum of the immunized rabbit used in Exp. 5 was heated for 20 minutes at 60° C. to destroy its complement.

Equal parts of this heated serum and an emulsion of B. T. "Rawlings" in saline were left in contact for 1 hr. at 37° C. The sensitized bacilli were centrifuged, the deposit washed in saline, and the resulting emulsion diluted in series from 1 in 10 to 1 in 1 million.

A mixture of normal human blood 1 part and 0.5% sodium taurocholate solution two parts, was then prepared and allowed to stand for 1½ hrs. 10 c.mm. of this preparation was added to an equal volume of each dilution of the emulsion of sensitized cells. At the same time, as a control, 10 c.mm. of normal salt solution was mixed with an equal volume of each bacterial dilution.

Both series were then incubated for 20 hrs. and plated.

It was anticipated that, if complement were still active in the blood-bile salt mixture, this would enable the already sensitized bacilli to be dissolved, and the higher dilutions of bacterial emulsion would be sterilized.

On plating, however, it was found that there was complete growth in all the dilutions up to 1 in 1 million, proving that the blood, when mixed with sodium taurocholate, was unable to "complement" the sensitized bacilli.

It is evident then that the anti-bactericidal action of sodium taurocholate depends on interference with the complement, and not on inhibition of the action of the amboceptor.

It is not suggested that the taurocholate can prevent the complementing and digestion of sensitized typhoid bacilli when the latter have been ingested by phagocytes. Such observations as have been carried out indicate that phagocytosis and intracellular digestion of typhoid

bacilli can both take place in contact with a 0.5% solution of sodium taurocholate in citrated normal salt solution, though the destructive action of the bile salt upon the blood elements renders the observation difficult and unsatisfactory. But short of interfering with phagocytosis, the "anti-complement" action of the bile salts may perhaps have an important rôle in typhoid fever and the production of "carriers."

It may be permissible to consider for a moment the conditions obtaining in—say—the third week of an attack of typhoid fever. The agglutinating power of the blood is now high and the clumped bacteria have been, to a great extent, filtered out of the general circulation. It is tempting to imagine them "held up" in considerable aggregations in the internal organs, such as the spleen, the liver and the adenoid tissue of the intestinal mucosa.

Probably the anchoring of "clumps" in these organs makes the work of phagocytosis both by leucocytes and tissue cells an easier task in some respects, but the ingestion of many virulent bacteria must also lead to the breaking down of leucocytes and, in all probability, the liberation of complement. In other words, at this time, there is probably an appreciable amount of extracellular solution of the typhoid bacilli; a surmise which is supported by the onset of the toxic symptoms characteristic of the later stages of the disease.

But while the liberation of complement leads, in most situations, to the extracellular solution of the already sensitized bacilli, the presence of bile at any given point would presumably prevent this solution, and enable even sensitized typhoid bacilli to survive and multiply. It is just in the positions where such an anti-bactericidal action of the bile is possible that foci of infection are found in typhoid carriers, *e.g.* throughout the hepatic area and in the mucosa and walls of the gall-bladder.

The hypothesis put forward, while perhaps too speculative to be of value in itself, may give point to the experiments here recorded, and emphasize the importance of further work on the possible rôle of the bile salts in typhoid fever and its sequelae.