COMPARATIVE STUDIES OF THE ACTION ON THE PNEUMOCOCCUS OF BILE ACIDS AND UNSATURATED FATTY ACIDS, FOUND IN BILE IN THE FORM OF SOAPS.

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Up to the present time, in studying the action of bile on the pneumococcus, only the bile acids and whole bile have been taken into consideration; the presence in bile of higher unsaturated fatty acids which are there in the form of soaps and in such a concentration that their part in the action of bile on the pneumococcus cannot be disregarded, has been entirely overlooked. Moreover, only the phenomenon of the solubility of pneumococci in bile or bile acids has been attentively considered, no attempt being made to determine which bile substance is most effective in its antiseptic action on the pneumococcus or other bacteria.

This question is not without significance when one takes into account that, before their elimination into the gall bladder, bile substances are present in the liver tissues and blood where they may play a very important rôle in the infectious diseases. With that in mind, I have studied the action of the conjugated bile acids, cholic acids, and unsaturated higher fatty acids found in bile in the form of soaps. As no pure commercial products were available, the substances used were prepared in this laboratory.

Preparation of Crystallized Bile.[1]

The fresh ox bile mixed with twice its volume of 95 per cent alcohol was allowed to stand in the cold room overnight. The precipitated mucins were separated by decantation and filtration and were washed in the filter three times with 95 per

[1] The preparation of the bile acids was carried out by methods on which those of Hammarsten (1) and Schryver (14) are based.

cent alcohol. The washings were added to the filtrate. The alcoholic bile was concentrated by distillation under diminished pressure, mixed with animal charcoal, and further evaporated on the water bath with the aid of an electric fan. The residue was dried *in vacuo* over sulfuric acid and extracted with hot absolute alcohol. The alcoholic filtrate was precipitated with ether. The precipitate was dissolved in water and further decolorized by animal charcoal and again extracted with absolute alcohol. To the alcoholic filtrate, anhydrous ether was added until permanent cloudiness developed and the fluid was allowed to stand overnight in a cold room. The white precipitate of bile salts was separated by decantation, washed with ether, and used for experiments as "crystallized bile," which is a mixture of conjugated bile acids and may contain a small amount of salts of fatty acids.

Preparation of Glycocholic Acid.

To the "crystallized bile," dissolved in water to the concentration of 2 per cent, a solution of neutral lead acetate was added while stirring. The precipitated lead glycocholate was filtered off and dried in a desiccator over calcium chloride *in vacuo*. The dry substance was suspended in hot alcohol and the lead was removed by sulfurated hydrogen. From the concentrated alcoholic filtrate, the free glycocholic acid was precipitated by ether and recrystallized from boiling water on cooling.

Preparation of Taurocholic Acid.

Taurocholic acid was prepared from ox bile freed from mucins by alcohol and from glycocholic acid by ferric chloride. After the evaporation of the alcohol, the acid was precipitated by basic lead acetate and purified in the same manner as glycocholic acid but recrystallized from alcohol by the addition of ether containing a little water.

Preparation of Cholic Acids.

A concentrated solution of sodium hydroxide was added to the fresh ox bile to make in it an 8 per cent solution of NaOH. The fluid, with the admixture of a little sand, was boiled over a gas flame through an asbestos wire gauze. While heating under a reflux condenser, the flame was regulated to avoid foaming into the condenser. The heating was interrupted at night. After 30 hours of boiling, the warm fluid was diluted with twice its volume of water and while still warm a dilute hydrochloric acid was slowly added while stirring the fluid with a glass rod. A rich, greyish white precipitate was formed and a viscid dark yellow mass of cholic acids in the form of small pasty granules which were gathered up on the end of the rod and transferred into a beaker; by that means, the larger part of the cholic acids was separated mechanically. The rest of the cholic acid was recovered by the extraction of the precipitate with hot acetone and added to the mass already isolated. After evaporation of the acetone, the dry residue was powdered and washed with cold water, redissolved in hot acetone, and filtered hot.

After evaporation of the acetone, the residue was dissolved in diluted ammonia and decolorized by animal charcoal. The acids were then precipitated from the filtered solution by the addition of diluted hydrochloric acid. The precipitate was washed with water and dried *in vacuo* over calcium chloride and soda-lime, dissolved in acetone, and filtered while hot. On cooling the concentrated solution, crystals of the cholic acids were precipitated and were then washed in a filter with a small amount of cold acetone. The filtrate and washings together yielded, after concentration, a further quantity of crude cholic acids which contained a mixture of cholic, choleic, and desoxycholic acids.

Separation of cholic, choleic, and desoxycholic acids was based on the difference in the solubility of their magnesium and barium salts. For this purpose, to the 1 per cent watery solution of sodium salts of crude cholic acids was added one-tenth its volume of 20 per cent solution of magnesium chloride and the mixture was heated for an hour on the water bath; a white precipitate was obtained which consisted of magnesium salts of choleic and desoxycholic acid while the magnesium salt of cholic acid remained in solution and the cholic acid was recovered by acidification of the filtrate. The choleic and desoxycholic acids were obtained by the decomposition of their magnesium salts suspended in water after acidification with hydrochloric acid. They were then dissolved in diluted ammonia, and 1 per cent solution of barium chloride was added. A precipitate was formed, and separated by filtration. This precipitate, washed with water, yielded, after acidification with diluted hydrochloric acid, the choleic acid, while from the filtrate after acidification, the desoxycholic acid was obtained.

The acids thus separated were further purified by conversion into their magnesium and barium salts and by recrystallization from hot acetone.

The Isolation of Fatty Acids from Ox Bile.

An amount of 4300 cc. of bile was collected from fresh gall bladders with all precautions to avoid contamination by fats from other tissues. The specific gravity of the fluid was determined as 1.03. The mucins, which are troublesome, were precipitated by alcohol, removed by filtration, and extracted repeatedly with 95 per cent alcohol. The alcoholic extracts were added to the filtered bile. After evaporation of the larger part of the alcohol by distillation under diminished pressure, 10 cc. per liter of 30 per cent potassium hydroxide were added and the fluid was extracted five times with petroleum ether until it was colorless and no residue remained after the evaporation of a sample.

By this means, the neutral fats and lipoids were removed from the bile in an amount of 22.65 gm. After that, the alkaline bile was acidified with diluted hydrochloric acid and again extracted several times with petroleum ether which now separated from the bile the fatty acids originally present in it in the form of soaps. The ethereal solution, washed with water and dried *in vacuo*, yielded a substance the larger part of which was crystallized in white needles with an admixture of an oily yellow fluid. The amount of this substance was 14.6 gm.,

and its iodine value of 50, determined by Wys' (16) method, proved that it contained a large amount of unsaturated compounds.

The separation of unsaturated fatty acids was accomplished by Varrentrapp's method based on the difference in the solubility of their lead salts. For this purpose, the fatty acids were dissolved in absolute alcohol; to this hot solution was added lead acetate dissolved in hot alcohol. After cooling, a white precipitate was formed which consisted of the lead salts of saturated fatty acids while those of unsaturated fatty acids remained in solution. The precipitate was washed with ether and the washings united with the filtrate which was concentrated and treated with a diluted hydrochloric acid and extracted with ether. The ethereal solution, washed with water, gave, after evaporation, a brownish yellow oily mixture in an amount corresponding to 0.17 per cent of whole bile and consisting of unsaturated higher fatty acids; the iodine value of this substance was 160, which indicates the presence not only of oleic but also of higher unsaturated fatty acids.

The oleic acid was separated from other more unsaturated fatty acids by means of barium salts. For this purpose, the mixture of unsaturated fatty acids was dissolved in a diluted ammonia and a solution of barium chloride was added while stirring. This precipitate was separated by filtration, washed with water, and partially dried between blotting paper and after that *in vacuo* over calcium chloride. The dry barium salts were dissolved in hot benzene containing 5 per cent of 95 per cent alcohol and allowed to stand overnight in the cold room. The white precipitate of barium oleate was formed; this was removed by centrifugalization, washed with alcoholic benzene, redissolved in hot alcoholic benzene, and decomposed by diluted hydrochloric acid. One obtained from this fraction an oily liquid, solidifying at 0°C. and melting at about 10°C.; its iodine value, determined by Wys' method, was 80, which approximates the theoretical value of oleic acid (90). Its amount corresponded to 0.07 per cent of whole bile. From the barium salts, which remained in solution in cold alcoholic benzene, one obtained after decomposition, purification, and drying *in vacuo*, a dark yellow oily liquid in an amount corresponding to 0.1 per cent of whole bile. Its iodine value was 174, which approximates that of linolenic acid (181). The fluid did not solidify at -6°C.

The fatty acids were further identified by the bromide test. While the fraction with an iodine value of 80 gave no solid bromides, the fraction with an iodine value of 178, brominated in ethereal solution acidified by acetic acid, gave crystallized bromides insoluble in petroleum ether and in cold benzene but almost entirely, although not completely, soluble in hot benzene. This reaction indicates that among the unsaturated fatty acids obtained from bile, there were present not only the acids with one and two, but also with more double bonds.

Preparation of Sodium Salts.

All the above mentioned bile acids and unsaturated fatty acids have been converted into sodium salts to bring out the action of anions. For this purpose, a

substance was dissolved in absolute alcohol, 3 drops of 1 per cent alcoholic solution of phenolphthalein were added, and a fresh alcoholic solution of sodium hydroxide was added drop by drop until a faint alkaline reaction appeared. The precipitated salts were washed with alcohol and ether. The further yield of salts was recovered from the concentrated and finally evaporated filtrate.

Comparison of the Action on the Pneumococcus of the Bile Acids and Unsaturated Fatty Acids.

In studying the action of the above mentioned substances on the pneumococcus, the highest (*a*) inhibitory and (*b*) bactericidal dilutions were determined.

The preliminary experiments showed the highest antiseptic dilution to be as follows: conjugated bile salts about 1:300; sodium choleate 1:1000; salts of unsaturated fatty acids 1:30,000. For the more precise experiments, the solutions of bile acid salts prepared were 5, 2.5, 1, 0.5, 0.166 per cent, and lower. These solutions were sterilized in the autoclave for 15 minutes at 120°C. under 15 pounds pressure.

1 cc. of a solution was added with all antiseptic precautions to the test-tubes containing 4 cc. of beef infusion broth to obtain the series of dilutions: 1:100, 1:200, 1:500, 1:1000, 1:2000, and so on up to the dilution 1:5000. To the control test-tubes, containing also 4 cc. of broth, was added 1 cc. of distilled water. The soap solutions were prepared in a parallel manner starting from 0.1 per cent solution up to 0.005 per cent to obtain the dilutions 1:5000, 1:10,000, 1:20,000, 1:30, 000, and so on up to 1:100,000.

For the comparative examination of different types and strains, one solution of salt (1 per cent solution of bile acids and 0.1 per cent solution of soaps) was made and it was added in corresponding amounts to a series of flasks each containing 200 cc. of broth to get the desired dilutions. Medium thus prepared was dispensed aseptically in the test-tubes, sterilized in the Arnold autoclave at 100°C. for 20 minutes, and incubated at 37°C. for 3 days to insure sterility and after that inoculated with pneumococci.

The medium used for all the experiments was beef infusion broth prepared as follows:

Beef chopped	1 lb.
Distilled water	1 kg.
Peptone	1.0 per cent.
Sodium chloride	0.5 " "
(pH 7.6)	

The reaction of the medium after the addition of the solution was only very slightly changed (pH 7.5).

(*a*) *Inhibitory Action.*—To the test-tubes containing corresponding dilutions of bile substances in broth, prepared as previously described, 0.2 cc. of an 18 hour pneumococcus culture was added and the tubes were incubated at 35°C. for 4 days. From those in which the broth remained entirely clear, or clouded for only a short time, transfers were made at different time intervals in an amount of one loopful in one, and of 0.2 cc. in the other series of test-tubes containing pure broth; the re-inoculated test-tubes were incubated under the same conditions and observed for 4 days. The results are given in Table I.

(*b*) *Bactericidal Action.*—The test-tubes containing exactly 4 cc. of broth were inoculated with 0.2 cc. of an 18 hour pneumococcus culture and incubated for 4 hours at 35°C. In this time, the growth was sufficient to make the broth distinctly cloudy. After that, 1 cc. of a corresponding solution of bile substances was added to get the same series of dilutions as in the first experiment. The test-tubes were replaced in the incubator and from time to time transplants were made from the tubes in which broth became clear.

TABLE I.

Action of Bile Substances on Pneumococcus Growth.

Substance.	Dilutions in which		
	Growth was entirely inhibited and the bacteria killed in less than 24 hrs.	Growth inhibited and bacteria alive after 24 hrs.	Growth partially inhibited.
Whole ox bile sterilized at 100°C. for 10 min.	1:10	1:50	1:100
Crystallized bile	1:300	1:500	1:1000
Sodium glycocholate	1:300	1:500	1:1000
" taurocholate	1:500	1:1000	1:2000
" cholate	1:1000	1:2000	1:3000
" choleate	1:1000	1:2000	1:3000
" desoxycholate	1:1000	1:2000	1:4000
" salt of unsaturated fatty acid with iodine value 80	1:30,000	1:40,000	1:70,000
Sodium salt of unsaturated fatty acid with iodine value 174	1:50,000	1:70,000	1:100,000

The bactericidal dilutions were found to be a little higher than the figures in the first column of Table I. The bactericidal action of higher unsaturated fatty acids and bile acids depends on the same phenomenon; that is, on the dissolving of pneumococci (compare Tables II and III) although the first are about 100 times stronger than the second (compare Table I). The reaction of the several types and

TABLE II.

Action of Sodium Taurocholate on Pneumococcus Growth.

(*a*) To test-tubes containing 4 cc. broth was added 1 cc. of corresponding solution; after that, 0.2 cc. of pneumococcus culture was inoculated.

Time.	Dilutions.							
	1:200	1:500	1:1000	1:2000	1:3000	1:4000	1:5000	Control.
hrs.								
4	−	−	−	−	±	+	+	+
10	−	−	−	±	+	++	++	++
24	−	−	−	−	±	+	++	++
48	−	−	−	−	−	±	±	+
in transplants made after								
hrs.								
4	−	+	+	+				
10	−	−	+	+				
24	−	−	+	+				
48	−	−	−	−	−	+	+	+

(*b*) Test-tubes containing 4 cc. broth were inoculated with 0.2 cc. pneumococcus culture and incubated at 35°C. for 4 hours; after that, 1 cc. of corresponding solution was added.

hrs.								
1	±	+	++	++	++	++	++	++
4	−	−	−	±	+	++	++	++
10	−	−	−	−	±	+	++	++
24	−	−	−	−	−	±	+	++
48	−	−	−	−	−	−	±	+
in transplants made after								
hrs.								
4	−	−	+	+	+	+	+	+
10	−	−	−	+	+	+	+	+
24	−	−	−	−	+	+	+	+
48	−	−	−	−	−	−	+	+

− = broth clear.
± = broth slightly cloudy from growth of the pneumococcus.
+ = broth distinctly cloudy from growth of the pneumococcus.
++ = broth very cloudy from growth of the pneumococcus.

TABLE III.

Action of Sodium Oleate on Pneumococcus Growth.

(*a*) To test-tubes containing 4 cc. broth was added 1 cc. of corresponding solution; after that, 0.2 cc. of pneumococcus culture was inoculated.

Time.	Dilutions.											
	1:5000	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:90,000	1:100,000	Control.
hrs.												
4	−	−	−	−	−	−	±	±	+	+	+	+
8	−	−	−	−	±	±	±	+	++	++	++	++
18	−	−	−	−	−	±	±	±	+	++	++	++
24	−	−	−	−	−	−	±	±	±	++	++	++
in transplants made after												
hrs.												
4	−	−	+	+	+	+	+					
18	−	−	−	−	+	+	+					
24	−	−	−	−	−	+	+	+	+	+	+	+

(*b*) To 4 cc. broth culture, incubated at 35°C. for 4 hours, was added 1 cc. of corresponding solution and further incubated.

min.												
30	−	+	+	++	++	++	++	++	++	++	++	++
hrs.												
1	−	±	+	+	++	++	++	++	++	++	++	++
2	−	−	−	+	+	+	++	++	++	++	++	++
4	−	−	−	−	−	±	+	++	++	++	++	++
10	−	−	−	−	−	−	−	+	++	++	++	++
12	−	−	−	−	−	−	−	−	+	++	++	++
24	−	−	−	−	−	−	−	−	±	+	++	++
in transplants made after												
hrs.												
1	−	+	+	+								
2	−	−	−	+	+	+						
4	−	−	−	−	−	+						
10	−	−	−	−	−	−	−	+				
24	−	−	−	−	−	−	−	−	+	+	+	+

− = broth clear.
± = broth slightly cloudy from growth of the pneumococcus.
+ = broth distinctly cloudy from growth of the pneumococcus.
++ = broth very cloudy from growth of the pneumococcus.

strains of the pneumococcus is different. Some strains are more or less sensitive to the action of the bile substances examined, and even in the culture of the same strain some cells are more resistant than others.

DISCUSSION.

In 1900, Neufeld (11) first drew attention to the solubility of the pneumococcus cell in bile. He attributed this action exclusively to the bile acids. In the report of his research on the hemolytic action of oleic acid (12) (1908) he says:[2] As regards their effect on the pneumococcus, the bile acid salts occupy an entirely isolated position. In particular it should be mentioned that neither soap nor concentrated solutions of potassium hydroxide, nor "sapotoxin" dissolve pneumococci. That this assertion is not entirely justified has been shown by Lamar (5) (1911) who, in his studies on the influence of unsaturated fatty acids on the solubility of the pneumococcus by immune serum, gave figures which are not far from those obtained in my experiments. The amount of higher unsaturated fatty acids I isolated was 0.17 per cent (crude substance) of whole bile; that is, they are present there in a dilution of about 1:600 in which they kill the pneumococcus in a few minutes, while the conjugated bile acids were found in ox bile by Hammarsten (1) and many others in a concentration of from 0.5 to 1 per cent; that is, they are present in bile in a dilution of from 1:200 to 1:100, in which they kill pneumococci after about 10 hours or more. In this case, the presence of higher unsaturated fatty acids in bile is more important than that of bile acids.

The presence of soaps of unsaturated fatty acids in bile should be taken into consideration not only by the bacteriologist but also by the physiologist and biochemist as these substances seem to be in a genetical relation to cholesterol and bile acids (Lifschütz (8)), and they may play a very important rôle in fat metabolism (Leathes (7)) and in jaundice.

In this connection there are only two suggestive articles; one by Trifanovsky (15), the other by Lassar-Cohn (6). The former (1874) found in the alcoholic extract from human bile, besides the bile acids, also the saturated higher fatty

[2] Neufeld and Händel (12), p. 581.

acids with an admixture of an oily substance which he believed to be oleic acid but did not study further. Lassar-Cohn (6) (1893) in the preparation of cholic acid found an oily substance the lead salt of which was soluble in ether, a fact which caused him to suggest that it was oleic acid; but he also did not undertake any further examination. By the method he used (boiling of bile with 6 per cent sodium hydroxide for 24 hours—Mylius' (10) method) not only the conjugated bile acids can be dialyzed but also the neutral fats and phospholipins. One cannot be sure then whether the oleic acid he obtained had its origin in the neutral fats or in the soaps of bile. The remarks found in different handbooks should therefore be corrected (Oppenheimer (13); Hoppe-Seyler (3)) that Lassar-Cohn (6) has detected (1893) the salts of oleic acid in bile. The same objection can be made to Mathews (9) who claims in his handbook that Hammarsten (2) has detected the soap of oleic acid in bile, as in the cited papers one finds that Hammarsten distinctly states that he obtained it by the hydrolysis of neutral fats of bile. No other significant articles or observations concerning oleic acid or the more unsaturated fatty acids in bile were found in the literature.

Kauftheil and Neubauer (4) (1924) have recorded a little lower bactericidal limit of desoxycholic acid (1:200) for the pneumococcus than in my investigations, which is possibly due to the fact that they used a different culture medium.

SUMMARY.

Ox bile contains the soaps of unsaturated higher fatty acids not only with one, but also with two and more than two double bonds, in a dilution of about 1:600. These substances, when isolated from bile, exert an antiseptic action on the pneumococcus in a dilution of approximately 1:50,000 and kill the organism in a dilution of 1:5000 in approximately 1 hour. As their action is, then, about 100 times stronger than that of the conjugated bile acids, their presence in bile should be considered, whereas hitherto it has been entirely overlooked not only by bacteriologists but by physiologists as well. Since the soaps of unsaturated fatty acids do dissolve the pneumococcus cell in broth culture, Neufeld's (12) conclusion is no longer valid.

In conclusion, I wish to express my appreciation and thanks to Dr. Augustus B. Wadsworth, Director of the Division of Laboratories and Research of the New York State Department of Health, Albany, whose kindness has made it possible for me to carry out these investigations.

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