

CXXVI. THE PREPARATION OF THE UN- CONJUGATED ACIDS OF OX-BILE.

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It has been known for some time that deoxycholic acid is able to bring about lysis of Pneumococci, and that its lytic action is about ten times as great as that of cholic acid. Nevertheless there has not hitherto been available a simple and reliable method of preparing deoxycholic acid which is sufficiently pure for use in bacteriological work. Of the principal methods of preparing deoxycholic acid, those of Pregl [1903] and Pregl and Buchtala [1911] are expensive and difficult to carry out. The method of Langheld [1908] is said by Wieland and Sorge [1916] to be impracticable. The method of Schryver [1912] is reliable but very lengthy, and for that reason is unsuitable for the regular production of deoxycholic acid. The method of Mair [1917] is simple, but the product, though sometimes satisfactory, in my experience is often so contaminated with higher fatty acid that it is quite useless for bacteriological work. Also the method has been found to be unworkable with some samples of ox-bile. Of the methods of preparing cholic acid, the simplest and most recent is that of Wieland and Weil [1912], but it has the disadvantage that only cholic acid is obtained, and that different procedures are necessary for summer bile and for winter bile.

This research was commenced with the object of finding a simple and reliable method of preparing deoxycholic acid suitable for bacteriological work, and in this paper two methods of preparation are described, one of which gives maximum yields of both deoxycholic acid and cholic acid, and the other is suitable for use when deoxycholic acid only is required and the yield of cholic acid is of secondary importance.

According to Wieland and Sorge [1916], choleic acid is a compound of deoxycholic acid with higher fatty acids. If this is crystallised from glacial acetic acid, the higher fatty acids are displaced, and a deoxycholic-acetic acid compound is obtained. This when pure melts at 145°. Pure deoxycholic acid, uncombined with fatty acid, melts at 173°. In this investigation the final substance arrived at was the deoxycholic-acetic acid compound, sometimes also called acetic-choleic acid.

METHOD I.

This method gives a maximum yield of both deoxycholic acid and cholic acid, and works successfully with ox-bile at any season of the year. Up to 10 litres of bile may be conveniently worked up at a time.

The principle of the method is first to convert the conjugated bile acids into the unconjugated form by hydrolysis of the bile with sodium hydroxide. The acids of the bile are then treated as a 10 % solution of their sodium salts by fractional precipitation with magnesium chloride solution. Magnesium deoxycholate is much less soluble in water than magnesium cholate, and also the magnesium salts of the higher fatty acids are less soluble than magnesium deoxycholate. Consequently the first precipitate, which is usually rejected, consists principally of useless magnesium soaps, which, if not removed, would interfere with the subsequent crystallisation of the second precipitate from 60 % acetic acid. The second precipitate, which is almost free from pigment, consists of the magnesium salts of most of the deoxycholic acid, and of some cholic acid. The bulk of the cholic acid remains in solution. The magnesium deoxycholate which has been precipitated is freed from admixture with cholic acid, and the free acid at the same time regenerated by crystallisation from 60 % acetic acid. The product is freed from fatty acid by converting it into the xylene-deoxycholic acid compound and this is reconverted into aceticcholeic acid by crystallisation from glacial acetic acid. Cholic acid is recovered from two sources, the solution which is left after the precipitation of the magnesium deoxycholate, and the 60 % acetic acid filtrate. From the former it is precipitated by acidification, decolorised with charcoal, and any deoxycholic acid still present precipitated by treating it as a 2 % solution of its sodium salts with magnesium chloride after Schryver's method. The magnesium cholate solution is acidified, and the precipitated cholic acid rubbed up with cold alcohol and filtered off. The cholic acid which is thrown out of solution on pouring the 60 % acetic acid filtrate into water is similarly rubbed up with cold alcohol in order to remove impurities which render very difficult the crystallisation of the crude material from hot alcohol in the ordinary way. This treatment also removes pigment and any remaining deoxycholic acid more efficiently than ordinary crystallisation from alcohol, the latter because the deoxycholic acid, which is present in an uncombined form or combined with acetic acid, is very easily soluble in cold alcohol and is easily removed by it, but in hot alcoholic solution it would combine with higher fatty acid to form a compound which is only moderately soluble in alcohol. After one treatment with cold alcohol, the cholic acid is recrystallised from hot absolute alcohol in the ordinary way.

Hydrolysis of the bile. For the purpose of hydrolysis, ox-bile obtained fresh from the slaughter-house is heated almost to boiling in a covered iron vessel for 20 hours with sodium hydroxide, 60 g. of this substance, dissolved in 100 cc. of water, being employed for each litre of bile. Any fluid lost by evaporation

is then replaced by the addition of water, and hydrochloric acid is added to the hot solution until a drop of it just fails to turn phenolphthalein pink. About 180 cc. of strong hydrochloric acid is required to neutralise each 60 g. of added soda, and if the solution is sufficiently hot and is carefully stirred, it can be added without precipitating any of the bile acids. After cooling, the neutralised solution is made definitely acid by the addition of hydrochloric acid with stirring to precipitate the bile acids. The precipitate is allowed to settle for a few hours; with different samples of bile it varies from a thick oil to a pasty mass, which becomes brittle on standing.

Separation of the greater part of the deoxycholic acid as a magnesium salt. The supernatant fluid is decanted from the pasty mass of hydrolysed bile acids, which is kneaded to free it from excess of water and mineral acid, and added to a boiling 10 % solution of sodium hydroxide, of which about 60 cc. is used for each litre of bile which is being worked up. The mixture is heated and stirred, more soda being added if necessary, until the solid material has dissolved. The excess of soda is then neutralised to phenolphthalein by adding dilute acetic acid to the vigorously stirred and boiling-hot solution. The solution is then diluted to 600 cc. for each litre of bile, which gives a solution of the solid material of the bile of 6–10 % strength. To this is added one-fiftieth of its volume of 40 % magnesium chloride solution, the mixture stirred, and heated for 1 hour at 90–100°. When the mixture is cold it is filtered, the supernatant liquor being decanted into the filter as completely as possible before the precipitate is thrown on to it. The precipitate (precipitate 1 a) is washed on the filter with a little cold water, and if it is in the slightest degree pigmented and greasy should be rejected, as in that case it is largely composed of uncrystallisable fatty substances. Sometimes, however, it is a white perfectly granular substance, and in that case it may be further treated in a way to be described. To the filtrate is added one-tenth of its volume of 40 % magnesium chloride solution, and the mixture heated, with occasional stirring, for 1 hour at 90–100°. A very heavy precipitate is produced, the solution setting almost to a paste. When it is cold, this is filtered off on the pump, filtration being quick and easy, and the precipitate is washed on the filter with several quantities of cold water, and pressed with a spatula until a perfectly white and only slightly moist substance is left on the filter (precipitate 1 b). The further treatment of the filtrate (filtrate 1 b) from which cholic acid and a further small amount of deoxycholic acid are obtained, is described below.

Crystallisation of the precipitated magnesium salts from 60 % acetic acid. Precipitate 1 b is dried at 100–110° until it can be crumbled to a powder. This is dissolved in four times its weight of boiling 60 % acetic acid and left for 2 days to crystallise out, crystallisation being aided if the mixture is stirred after standing a day. The crystals are then filtered off and washed on the filter with small quantities of cold 60 % acetic acid till free from pigment (precipitate 1 c).

Precipitate 1 a usually consists of fatty material which, if it were not

removed, would prevent the second precipitate of magnesium salts from crystallising from acetic acid. Occasionally, however, when less of these fatty substances is present in the bile, it is a white, perfectly granular, rather bulky precipitate, and in this case it should be treated in the same way as precipitate 1 *b*. As, however, it contains a certain amount of inorganic matter, its solution in 60 % acetic acid must be filtered whilst boiling-hot. Any material crystallising from the filtered solution may then be added to precipitate 1 *c*.

Removal of fatty acid by boiling with xylene. The material crystallised from the 60 % acetic acid is dried for 1 hour at 100°, powdered, and boiled vigorously under reflux in a conical flask for about 3 hours with eight times its weight of xylene. At the end of this operation the mixture is allowed to become perfectly cold, and the xylene-deoxycholic acid compound filtered off on the pump, washed with a little cold xylene and well drained (precipitate 1 *d*). The filtrate contains only some fatty acid and a trace of bile acid and pigment, and the xylene is easily recovered from it by distillation.

Conversion of the xylene-deoxycholic acid compound into deoxycholic acid by crystallisation from glacial acetic acid. The xylene-deoxycholic acid compound (precipitate 1 *d*) is dried for 1 hour at 100–110°, dissolved in four times its weight of glacial acetic acid, the solution boiled for half a minute and left to crystallise. After standing 24 hours the crystals (precipitate 1 *e*) are filtered off, washed on the filter with a very little cold glacial acetic acid, and well drained. They are colourless, and consist of nearly pure deoxycholic acid combined with acetic acid (acetic-choleic acid). The m.p. is usually 137–141° or higher, and the material is pure enough for bacteriological purposes. It may be further purified by recrystallisation from four times its weight of glacial acetic acid.

From the mother liquors of any further crystallisations from glacial acetic acid, a further crystallisation of less pure deoxycholic acid, m.p. 130–135°, can be obtained by evaporation to about one-third of their volume.

Treatment of filtrates.

The filtrate 1 b from the precipitation of the magnesium salts contains the greater part of the cholic acid and pigment of the bile, and a certain amount of deoxycholic acid. It is acidified with dilute hydrochloric acid and stirred, the precipitate allowed to settle, collected in one mass, kneaded to free it from water and mineral acid, and added to a hot 10 % solution of sodium hydroxide, about 30–40 cc. of a 10 % solution being required for each litre of bile. The solution is warmed and stirred, more soda being added if necessary, until the solid material has all been converted into sodium salts and dissolved. To the very hot solution dilute acetic acid is added, with careful stirring, until it is only weakly alkaline, and then 20–25 g. of charcoal for each litre of bile is stirred into the solution. The mixture is now evaporated to dryness and finally dried in the air-oven at 100–110°. The dry mass is then powdered, and extracted with methylated spirit for 12–15 hours in a Soxhlet apparatus to remove the sodium salts of the bile acids.

The methylated spirit extract is evaporated to dryness, and a 2 % aqueous solution of the residue is made. To this solution, which is only lightly coloured, one-tenth of its volume of a 40 % solution of magnesium chloride is added, the mixture stirred, and heated for 1 hour at 90–100°. A precipitate of magnesium deoxycholate is produced, and is filtered off when the mixture is cold. This precipitate is treated in the same way as the precipitate 1 *b* which was obtained at an earlier stage of the preparation, and both deoxycholic acid and cholic acid can be obtained from it.

The filtrate is acidified with acetic acid, stirred, allowed to stand overnight and the precipitate collected, kneaded to free it from water, and rubbed up in a strong-walled beaker to a thin cream with 2–3 volumes of methylated spirit, sufficient being used to leave a thin layer of clear pigmented fluid above the material when it is allowed to settle. The mixture is left overnight in the ice-chest, and the finely crystalline mass is then filtered off and washed with small quantities of cold methylated spirit, on the filter, until a perfectly white powder is left. This is impure cholic acid, m.p. 185–190°. It is easily purified by two recrystallisations from absolute alcohol.

The filtrate 1 c from the crystallisation from 60 % acetic acid contains a large amount of cholic acid. It is poured into about ten volumes of water, left overnight, the supernatant liquor decanted off, the precipitated solids kneaded to free them from water, and rubbed up to a cream with methylated spirit in the same way as the precipitate whose treatment is described in the previous paragraph. It is most important that the methylated spirit cream should not be heated or the product will be much less pure. On filtering off the material after standing overnight, and after careful washing on the filter with small quantities of cold methylated spirit, a perfectly white powder is obtained which is impure cholic acid, m.p. 190–195°.

METHOD II.

This is shorter than method I, but the yield of deoxycholic acid is rather less, and only a small amount of cholic acid is obtained. The method is usually reliable, but occasionally with some samples of bile it may be impossible to obtain a satisfactory crystallisation from acetic acid of the crude product from the hydrolysis. In such cases method I must be used. Up to 10 litres of bile may be conveniently worked up at a time.

The principle of this method is very similar to that of the method of Mair [1917], the separation of the two bile acids depending upon the fact that cholic acid is easily soluble in 60 % acetic acid or glacial acetic acid, whilst deoxycholic acid is comparatively insoluble in these solvents. The deoxycholic acid is freed from admixture with fatty acid by converting it into the xylenecholeic acid compound, and cholic acid is obtained from the second and third acetic acid filtrates, and purified by treatment with alcohol, on the lines described in connection with method I.

Hydrolysis of the bile. This operation is carried out in the same way as in method I, except that the bile acids are precipitated from the neutralised

solution after hydrolysis by adding glacial acetic instead of hydrochloric acid. This avoids the presence of a dangerous amount of mineral acid when the bile acids are subsequently heated with 60% acetic acid. About 20 cc. of glacial acetic acid are required for each litre of bile, and whilst it is being added the solution is stirred round with a glass rod, on which the precipitated bile acids are collected into a single pasty mass which can be easily removed.

Crystallisation of the crude hydrolysed bile acids from acetic acid of about 60% strength. As much fluid as possible is kneaded out of the precipitate, and it is weighed and dissolved by heating in rather more than an equal weight of glacial acetic acid and left to crystallise out. After standing for 4 days the crystals are filtered off on the pump, freed from pigment by washing on the filter with small quantities of cold 60% acetic acid, and well drained. The filtrate is rejected.

Crystallisation from glacial acetic acid to remove cholic acid and inorganic impurities. The material crystallised from 60% acetic acid is dissolved in three parts of glacial acetic acid, and the boiling-hot solution is filtered by suction through a Büchner filter of ample size. To ensure easy filtration the filter funnel should be heated beforehand, and the filter paper moistened with hot glacial acetic acid. After standing for 24 hours, the material which has crystallised out is filtered off by suction, washed with a very little cold glacial acetic acid on the filter, drained, and dried for 1 hour at 110°. The filtrate (2 *b*) is treated as described below. The dried material is further treated by the procedure described in method I, commencing at the stage where the precipitate 1 *c* is boiled with eight times its weight of xylene. The final product is deoxycholic acid, combined with acetic acid, m.p. 141–144°, similar to that obtained by method I. The filtrate (filtrate 2 *b*) is treated in exactly the same way as the filtrate 1 *c* obtained from the crystallisation from 60% acetic acid in method I. Impure cholic acid, m.p. 190–195°, is obtained, and is finally purified by recrystallisation from absolute alcohol.

YIELDS.

The yields per litre vary considerably with different samples of bile.

Method I.

Deoxycholic acid	m.p. 141–144°	10–13 g.
Deoxycholic acid ¹	m.p. 130–135°	1–2 g.
Cholic acid ²	m.p. 185–195°	19–23 g.

Method II.

Deoxycholic acid	m.p. 141–144°	6–8 g.
Deoxycholic acid ¹	m.p. 130–135°	1–2 g.
Cholic acid ²	m.p. 190–195°	6–8 g.

¹ Second crystallisations from later glacial acetic acid mother liquors.

² Weighed before final purification by recrystallisation from absolute alcohol.

PREPARATION OF UNCOMBINED DEOXYCHOLIC ACID
FROM ACETIC-CHOLEIC ACID.

As the deoxycholic-acetic acid compound produces lysis of Pneumococci perfectly satisfactorily, it is unnecessary to remove the acetic acid from material which is to be used for bacteriological work. It is, however, worth while mentioning two methods of doing this which have proved satisfactory.

1. *By digesting acetic-choleic acid in dilute sodium hydroxide solution.* Pure acetic-choleic acid is dissolved in 100 volumes of *N*/10 sodium hydroxide solution, and the solution heated under reflux for 5 hours on the water-bath. After cooling the solution is acidified, with careful stirring, with the least possible amount of dilute hydrochloric acid. The precipitated deoxycholic acid is filtered off, washed on the filter with water, and dried *in vacuo* at room temperature for 1-2 days, and then for a few hours at 100-110°. Very careful drying is essential in order to remove all trace of hydrochloric acid: M.P. of product 169-172°.

2. *By distilling off the acetic acid in a current of steam.* Pure acetic-choleic acid is finely powdered and poured into five volumes of boiling water in a flask, and a strong current of steam is blown through the mixture for 5 hours. The acetic-choleic acid first changes into a viscous yellow fluid, which, as the acetic acid is removed, should form a powdery suspension, which collects into hard lumps.

After 5 hours the mixture is cooled, the deoxycholic acid filtered off and dried *in vacuo* as before: M.P. of product 169-172°.

The deoxycholic acid thus prepared can be crystallised from alcohol, but the procedure involves considerable loss, owing to the great solubility of the material.

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