THE PREPARATION OF THE UNCONJUGATED ACIDS OF OX-BILE.

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It has been known for a long time that ox-bile, on hydrolysis with alkalis, yields, in addition to the chief product, cholalic acid, at least two other acids, viz. choleic and deoxycholeic acids. Nevertheless, up till quite recently, no satisfactory method of separation of these acids has existed, and it has been impossible to form any conception as to the relative quantities which are produced when ox-bile is hydrolysed with caustic alkalis. As large quantities of bile-acids were required for the purposes of various investigations, a research was commenced some time ago, with the object of obtaining a method for preparing the unconjugated acids in a satisfactory manner. When the main principles of the separation described in this paper had been already evolved, a publication by Pregl and Buchtala[1] appeared, in which a method was described by means of which the three unconjugated bile-acids could be obtained in a satisfactory yield. It is of interest to note, however, that the method of Pregl and Buchtala, and the method described in this paper differ from one another in almost every detail.

Preparation of the crude hydrolysis products.

For the purpose of hydrolysis, ox-bile, obtained quite fresh from the slaughter-house, is heated in an iron digester fitted with a reflux condenser for 30 hours with sodium hydroxide, 170 grams of this substance dissolved in 300 c.c. of water being employed for 2·5 litres of bile, the quantity generally worked up in each hydrolysis. When this operation is complete, the hydrolysis mixture is diluted with twice the volume of water, and then acidified whilst still warm with an excess of dilute hydrochloric acid, the mixture being

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[1] *Zeitsch. physiol. Chem.* LXXIV. p. 198. 1911.

vigorously stirred after each small addition of acid. The crude unconjugated bile-acids separate out for the most part in the form of a very viscid oil, which on cooling forms a thick pasty mass. A small amount of acid also separates in a granular form. After standing over night, the whole is filtered off, and washed free from the excess of mineral acid by kneading for some time with distilled water. It is then dried on a water-bath after which it can be readily powdered. It is then dissolved up in excess of dilute ammonia, the solution being so diluted that it does not contain more than 5 % ammonium salt. From this some of the pigment is removed by boiling for about ten minutes with animal charcoal, which is then filtered off. The acid is precipitated from the filtrate by dilute hydrochloric acid. The precipitate thus obtained, after washing with water, is dried *in vacuo* over calcium chloride and soda-lime. It is removed from the desiccator from time to time, the surface lumps are powdered, and the lower layers of acid are exposed. The more completely the water is removed, the more readily can the mass be powdered; until finally, the whole can be obtained in a completely granular form.

Up to now, the process of preparation is practically the same as that employed by most of the earlier investigators. From this point onwards, however, the method has been entirely altered.

Instead of recrystallising from alcohol from which a crude cholalic acid separates only very incompletely and a large amount of uncrystallisable mother liquor is obtained, the crude acids are recrystallised from hot acetone. The powder is added to the boiling solvent until the latter is saturated. It is then filtered off when hot from small particles of charcoal and other insoluble impurities, and allowed to cool. After standing the crystals which separate are filtered off, and rapidly washed with the use of the pump with small quantities of cold acetone, until they are nearly free from the mother liquors. The filtrates and washings are then united and concentrated and allowed to cool until another crop of crystals separates. These are also washed, and the washings and mother liquors are again concentrated, and the process is repeated until a thick syrupy mother liquor is obtained from which only a few crystals are obtained after prolonged standing. By this process more than 80 % of the crude acid is obtained in the form of crystals which possess only a light greenish tinge.

Separation of cholalic, choleic and deoxycholeic acids from the crude crystalline mixture.

The next process depends upon the difference in the behaviour of the magnesium salts of these three acids. The crude acids are suspended in alcohol and the alcoholic solution is made just alkaline to phenol phthalein, the suspension of the acids in alcohol being kept warm during the addition of normal sodium hydroxide from a burette. As soon as a slight excess of alkali has been added, the alcohol is evaporated off on a water-bath, and the sodium salts are then taken up with water, so much of this solvent being used that 100 c.c. corresponds to one gram of the crude crystalline acids. A solution containing approximately 1 °/₀ of sodium salts is thereby obtained. This is filtered, and is generally faintly alkaline to phenol phthalein. The pink colour is destroyed by the addition of a few drops of very dilute acetic acid. The solution is then mixed with one-tenth of its volume of 20 °/₀ magnesium chloride solution. A perfectly clear mixture is thus obtained. If this be heated on a water-bath, a bulky crystalline precipitate gradually separates, and the whole mixture is sometimes thereby converted almost into a paste. After heating for about one hour, *the mixture is allowed to cool*, and the precipitate which consists of the magnesium salts of the greater part of the choleic and deoxycholeic acids, with only relatively small quântities (not more than about one-fifth of the total) of cholalic acid, is then filtered off. Sodium cholate behaves quite differently from the sodium salts of the mixed acids when its solution in water is treated with magnesium chloride solution. When a 1 °/₀ solution of the cholate is warmed on a water-bath with one-tenth the volume of 20 °/₀ magnesium chloride, it remains perfectly clear. A 5 °/₀ solution, on the other hand, when treated in the same way, yields a precipitate of fine silky needles, which, however, *redissolve on cooling.* If the precipitate is filtered off from the hot solution, and is allowed to stand exposed to the air, it rapidly forms a deliquescent mass. For this reason, the mixture from which the magnesium salts of the mixed acids have separated, is allowed to cool before filtering off the precipitate.

In spite of these facts, the treatment of 1 °/₀ solutions of the mixed sodium salts by the process above described does not lead to a complete separation of the cholalic from the other two acids. As already mentioned, about one-fifth of the cholalic acid is carried down with the magnesium salts of the two other acids. Furthermore, the magnesium salts of the other two acids are not quite insoluble.

Treatment of the magnesium salt precipitate. The precipitate, after the mixture has cooled, is filtered off by the pump, and the mother liquor is sucked off as completely as possible. The free acids are regenerated from this precipitate, by thoroughly rubbing up in a mortar with an excess of dilute hydrochloric acid. After standing, the acids get quite granular, and are then filtered off with the pump, and washed thoroughly with water. After drying *in vacuo* they are converted into sodium salts by suspending in alcohol and adding normal sodium hydroxide solution until the mixture is permanently alkaline to phenol phthalein. After evaporating off the alcohol, the sodium salts are taken up in water, so much solvent being added as to make up an approximately 2 °/₀ solution which is then, if alkaline, made just neutral to phenol phthalein, by the addition of very dilute acetic acid. Barium chloride in 20 °/₀ aqueous solution is then added in excess with continual stirring. A precipitate is produced, which is at first oily, but which on standing over night becomes granular and the sides of the vessel become often (but not always) coated with radiating groups of crystals. The precipitate produced does not consist, apparently, entirely of barium salts, for the sodium salts of both choleic and deoxycholeic acids are precipitated from solution in an oily form by the addition of salts, such as sodium chloride and ammonium sulphate; in this respect they differ from the sodium salt of cholalic acid, which is not precipitated in this way. The choleic acid, however, appears to be precipitated as barium salt in the above described process and it can be freed from the sodium salt of the deoxycholeic acid by thoroughly washing with water. Accordingly, after the barium chloride has been added in excess to the 2 °/₀ solution of sodium salts (about one-fifth of the volume of 20 °/₀ $BaCl_2$ is necessary) and the mixture has stood for about 16 hours, the precipitate is filtered off on a pump. As the filtration is somewhat slow, it is best to syphon off the supernatant liquid on to the filter, and as soon as this has passed through, to throw on to the filter the pasty mess from the bottom of the vessel in which the precipitation has been carried out. As soon as the precipitate is freed as completely as possible from the mother liquors, it is removed from the filter and thoroughly ground up with water in a mortar and again filtered. This process is repeated about three times, until the barium choleate is freed almost entirely from contaminations with the deoxycholate. On treating it then in a mortar, with dilute hydrochloric acid in excess, a nearly pure preparation of choleic acid is obtained, which after washing and drying *in vacuo* melts only one or two degrees below the melting point of the pure preparation.

The mother liquors and washings from the barium salt are then acidified, and a precipitate consisting chiefly of deoxycholeic and cholalic acids is obtained. These, after washing, are converted into sodium salts; to a 1 °/₀ solution of these is added one-tenth the volume of 20 °/₀ magnesium chloride solution, and the mixture is heated on a water-bath. The precipitate in this case consists chiefly of the deoxycholeate, mixed with only small quantities of cholalate. The mother liquor from the magnesium salt precipitate is concentrated on a water-bath to about one-fifth of the bulk when a further small quantity of deoxycholeate separates and is filtered off *when the solution is cool.* The filtrate from this is added to the filtrate from the first magnesium salt precipitate after the latter has been concentrated (see below).

Treatment of the filtrate from the first magnesium salt precipitate. The filtrate is concentrated on a water-bath to about one-fifth of its bulk, and when cool, the crystals which remain undissolved, and which consist almost entirely of deoxycholeate, are filtered off. The filtrate, to which is added the concentrated filtrate from the second magnesium salt precipitate, is then acidified with excess of dilute hydrochloric acid which is added slowly with constant stirring. The precipitate, which is thus formed, is at first somewhat resinous, but rapidly becomes crystalline on standing. It consists of cholalic acid, contaminated only with small quantites of the two other acids.

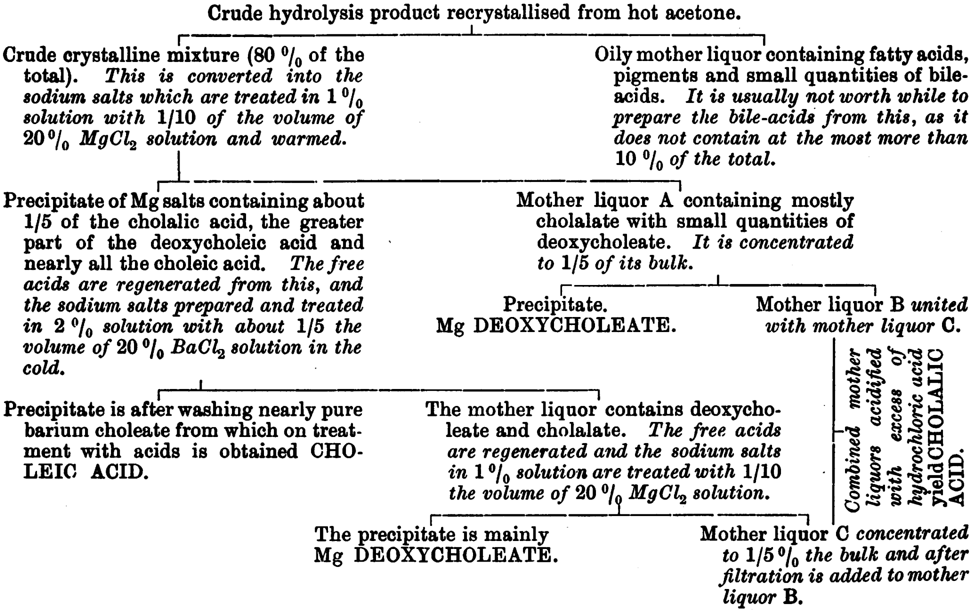
By the above processes, the crude bile-acids are separated into three main fractions, containing respectively the crude cholalic, choleic and deoxycholeic acids, from which, with very small loss, the pure acids can be readily prepared by the methods described below.

The general method is schematically indicated in the accompanying table (p. 270).

Purification of the crude separated acids.

Cholalic acid. This acid is obtained in the form of a yellowish crystalline mass. After washing and drying *in vacuo* it is dissolved in hot alcohol, from which the greater part separates on cooling. From the mother liquor, further crops of crystals are obtained. An appreciable portion, however, remains behind in the mother liquors and

Scheme for separation of the unconjugated bile-acids.



Crude hydrolysis product recrystallised from hot acetone.

Crude crystalline mixture (80 $^{0}/_{0}$ of the total). *This is converted into the sodium salts which are treated in* 1 $^{0}/_{0}$ *solution with* 1/10 *of the volume of* 20 $^{0}/_{0}$ *$MgCl_2$ solution and warmed.*

Oily mother liquor containing fatty acids, pigments and small quantities of bile-acids. *It is usually not worth while to prepare the bile-acids from this, as it does not contain at the most more than* 10 $^{0}/_{0}$ *of the total.*

Precipitate of Mg salts containing about 1/5 of the cholalic acid, the greater part of the deoxycholeic acid and nearly all the choleic acid. *The free acids are regenerated from this, and the sodium salts prepared and treated in* 2 $^{0}/_{0}$ *solution with about* 1/5 *the volume of* 20 $^{0}/_{0}$ *$BaCl_2$ solution in the cold.*

Mother liquor A containing mostly cholalate with small quantities of deoxycholeate. *It is concentrated to* 1/5 *of its bulk.*

Precipitate. Mg DEOXYCHOLEATE.

Mother liquor B *united with mother liquor* C.

Combined mother liquors acidified with excess of hydrochloric acid yield CHOLALIC ACID.

Precipitate is after washing nearly pure barium choleate from which on treatment with acids is obtained CHOLEIC ACID.

The mother liquor contains deoxycholeate and cholalate. *The free acids are regenerated and the sodium salts in* 1 $^{0}/_{0}$ *solution are treated with* 1/10 *the volume of* 20 $^{0}/_{0}$ *$MgCl_2$ solution.*

The precipitate is mainly Mg DEOXYCHOLEATE.

Mother liquor C *concentrated to* 1/5 $^{0}/_{0}$ *the bulk and after filtration is added to mother liquor* B.

refuses to separate in a crystalline form. To the last mother liquors is added therefore excess of caustic alkali, and the mixture is warmed on a water-bath for a short time, to hydrolyse any ester formed by the action of the alcohol on the acid. The alcohol is then evaporated off on a water-bath, the residue taken up in water, and the acids are precipitated from the solution of the sodium salts with excess of dilute hydrochloric acid. These, after drying, are converted into sodium salts, and a large excess of barium chloride (20 $^{0}/_{0}$ solution) is added to a 2 $^{0}/_{0}$ solution of the latter. A small oily precipitate is thus produced, which contains choleate and deoxycholeate. The quantities of these acids are so small, that it is not worth while to recover them, especially as they are intermixed with small amounts of resinous matter and they can only be purified with considerable difficulty. On acidifying the filtrate from the barium precipitate, cholalic acid is precipitated, which can be purified by recrystallisation from alcohol. From the very last mother liquors, a further quantity of crystalline cholalic acid can be obtained by converting the acids into sodium salts (by adding excess of caustic alkali to the mother liquors, warming, and evaporating off the excess of alcohol), acidifying the aqueous solution of the same in the presence of ether; the freshly precipitated acid readily dissolves in the ether, and separates in crystalline form a concentration of the solvent. Quite 90% of the crude acid can thus be obtained in crystalline form, with the M.P. 197°. It is generally slightly coloured. To recrystallise further it has been found convenient to convert the acid into sodium salt, and to precipitate the acid from the dilute solution of this salt by hydrochloric acid. The precipitated acid, after drying, readily dissolves in hot alcohol, from which most separates on cooling. This method has been found more convenient than direct recrystallisation from alcohol, for the acid, as it separates from this alcohol solvent with alcohol of recrystallisation, requires a large amount of liquid for re-solution.

Choleic acid. This acid, as it is obtained directly from the barium salt by rubbing up the latter with excess of dilute hydrochloric acid, is already nearly pure, and melts often at 184° (after drying at 110°), which is only two or three degrees below the melting point of the pure acid. It is best purified by recrystallisation from hot acetone. When, however, large quantities have been prepared, inconveniently large amounts of solvent are necessary to completely dissolve the product. In this case, alcohol has been used for recrystallisation. A pure product separates. A relatively large amount remains in this case in the mother liquors (perhaps one-third). These are therefore warmed with excess of caustic alkali on the water-bath, to hydrolyse the ester formed by the action of the alcohol, the excess of this solvent is evaporated off, the residual sodium salts are taken up with water, and the free acids are precipitated from this solution. These are, after washing and drying *in vacuo*, recrystallised from acetone. The first crops of crystals obtained are pure. The crops from the mother liquors are recrystallised until the products have the correct melting point. The yield of pure acid obtained by this method is very satisfactory.

Deoxycholeic acid. This acid is obtained from the magnesium salt by grinding it up with excess of dilute hydrochloric acid. The product thus obtained, after recrystallising from acetone, in which it is easily soluble, melts, after drying at 110°, at about 140°, with evolution of solvent of recrystallisation. Fractional crystallisation both of free acid from ethyl acetate and barium salt from water, reveals the fact that the product is not free from cholalic acid. On purifying it, however, by the method used by other investigators, viz. by recrystallisation from glacial acetic acid, a product melting at 145° is obtained. The final purification of this acid involves a larger loss than that resulting from the purification of the other acids.

Examination of the oily residue obtained after recrystallisation of the crude hydrolysis products from acetone.

In view of a recent discovery of a new bile-acid in ox-gall stones by Hans Fischer[1], and called by him lithocholic acid, a considerable amount of time was expended on the careful examination of the various mother liquors. No trace, however, of an acid corresponding with Fischer's acid has been discovered, and it appears probable that this is a pathological product which does not exist in normal bile. The last mother liquors from the acetone recrystallisation of the crude acids which were of dark green colour, were examined in the following way. The oily liquid was taken up with ether, and the ethereal solution was rapidly dried over anhydrous sodium sulphate and allowed to stand in a cold room. No crystals separated. The ethereal solution was then evaporated to a small bulk and the concentrated solution was then dropped, with continual stirring, into a large bulk of light petroleum. A pasty mass separated, which was gently warmed to make less viscid, and shaken up several times with further quantities of light petroleum.

On evaporating off the petroleum a residue was left behind, which on standing soon set to a mass of soapy crystals. These consisted of fatty acids.

The part insoluble in petroleum was then dissolved in methyl alcohol, and neutralised with a methyl alcoholic solution of barium hydroxide. A highly pigmented precipitate was formed, from which, on treatment with acids, a very dark yellowish green acid was obtained, which on drying *in vacuo* appeared almost black. This became pasty when treated with various solvents such as alcohol, acetone, ether, etc., but did not readily dissolve even on heating. This acid appears to be related to the bile pigments and was not further examined.

The greater part of the barium salts remained, however, in solution in methyl alcohol. The methyl alcohol was evaporated off and a thick syrupy mass was obtained. This on rubbing up with acetone was converted into a light yellow granular powder. The acetone dissolved up a small quantity of substance, which was obtained in the form of a thick oil, which did not crystallise even after prolonged standing. It gave the cholesterol reaction with chloroform and sulphuric acid.

The barium salt was treated with excess of dilute hydrochloric acid in the presence of warm ethyl acetate, which dissolved up the acid thus

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[1] *Zeitsch. physiol. Chem.* LXXIII. p. 234. 1911.

set free. The ethyl acetate solution was then dried over sodium sulphate, and evaporated to a small bulk. On the addition of light petroleum, a glassy acid separated which is probably identical with an acid of similar character isolated by Langheld[1]. This acid was submitted to examination; it is not necessary to go into great detail as to the method; suffice it to say, that by means of the magnesium salt and barium salts, by the method applied to the crude crystalline acids described above, it was possible to isolate from this product, cholalic, choleic and deoxycholeic acids. The yield of cholalic acid obtained was 20 °/$_0$ of the total, but this does not represent the maximum obtainable, as appreciable quantities of the material were used up in preliminary experiments. The purification of the other acids was attended with great loss, owing to the presence of small quantities of resinous matter.

Yield of the different products.

From 10 litres of bile the following amounts of the various products were obtained:

Cholalic acid	225 grams*	Obtained from 350 grams of crude crystalline acids.
Choleic acid	75 ,, *	
Deoxycholeic acid	40 ,, *	
Fatty acids	20 ,,	Obtained from the mother liquors of the acetone crystallisation.
"Pigment" acids	12 ,,	
"Glassy" acid	35 ,,	

* Quantities weighed before recrystallisation.

Pregl and Buchtala found in the bile obtained in summer from the Graz slaughter-houses the following percentages of the total yield of crude acids of the various products. Fatty acids 10 °/$_0$, cholalic acid 51·2 °/$_0$, choleic acid 11·9 °/$_0$, deoxycholeic acid 13·5 °/$_0$, non-crystallisable substances 12·6 °/$_0$. It is of interest to note that from the London bile, larger percentages of choleic than deoxycholeic acid were obtained, the reverse of what was found in the Graz bile.

SUMMARY.

(1) A method is described by means of which the unconjugated acids from ox-bile can be readily prepared in large yields.

(2) The crude acids are first recrystallised from hot acetone, and over 80 °/$_0$ are obtained directly in crystalline form.

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[1] *Zeitsch. physiol. Chem.* XXII. p. 396.

(3) From the crude crystalline mixture, the greater part of the cholalic acid can be separated by heating a 1 % solution of the sodium salts with one-fifth the volume of 20 % magnesium chloride on a water-bath. Most of the choleic and deoxycholeic acids separate as magnesium salt, the greater part of the cholalate remaining in solution.

(4) From the mixture of the choleate and deoxycholeate (containing only a relatively small quantity of the cholalate) the choleate can be separated as an insoluble barium salt. After this precipitation, the deoxycholeic acid can be separated from the greater part of the still adhering cholalic acid by reconversion into its magnesium salt (as under heading 3).

(5) From the mother liquors of the acetone crystallisation of the crude acids, the following products could be isolated—(*a*) fatty acids, (*b*) "pigment" acids, (*c*) "glassy" acid. From this latter acid, cholalic, choleic and deoxycholeic acids could be obtained.

(6) No trace was found of the "lithocholic" acid, recently isolated by Hans Fischer from ox-gall stones. It appears possible that this is essentially a pathological product.

It is proposed to apply the above method to the isolation of the acids from biles of other sources.