CXXXVII. A CRITICAL STUDY OF THE METHODS OF ESTIMATING CHOLESTEROL AND ITS ESTERS IN TISSUES, PART II.

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THE methods in use for the estimation of cholesterol and its esters in tissues are liable to many quite distinct sources of error. Errors may arise through faulty sampling, unsuitable methods of preparing the tissue for extraction, inadequate extraction, incomplete hydrolysis of esters, and lastly through defects inherent in the methods used for estimating the cholesterol in the extracts. The errors to which the gravimetric and colorimetric methods of estimating sterols are liable have been already fully discussed in Part I by Gardner and Williams [1921] and by Gardner and Fox [1921].

In this paper we propose to deal with the other sources of error and more particularly that due to incomplete hydrolysis of the esters.

Sampling.

It is not safe to cut off a piece of a weighed organ for analysis and assume that the percentage of sterol found gives a representative value for the whole. The whole organ should be finely minced and thoroughly pulped in a mortar and an aliquot portion taken. Even this is not safe in the case of tissues containing much intercellular fat. For example, two aliquot portions of a very carefully pulped mixture of several suprarenals gave 3.46 % of ether extract and 0.334 % of cholesterol in one and 3.40 % and 0.305 % in the other.

If the necessary apparatus is available greater uniformity of the material can be obtained by freezing the pulp and grinding the frozen mass. The most satisfactory method, however, is to extract, when possible, the whole of the pulped organs, and make the extract up to known volume and then take an aliquot portion for analysis. This is, however, rather troublesome in the case of large organs such as liver.

Preparation of the material for extraction.

If the material is to be directly extracted by ether or by any solvent immiscible with water, it must be dried. Unsuitable methods of drying are

fruitful sources of error. Drying by heat, particularly at 100°, is quite unsuitable. It has long been recognised that material dried in this way yields only a portion of its ether-soluble constituents to direct extraction by ether, though alcohol is much more effective. Even when a complete extraction has been effected, the ether extract finally obtained is always deep brown in colour; and in the estimation of the free cholesterol by digitonin the colouring matter is sometimes carried down with the cholesterol digitonide precipitate and is difficult to get rid of without danger of dissolving some of the digitonide itself.

The drying of the ether extract itself by too prolonged heating also appears to modify the ratio of free to bound cholesterol.

Thaysen [1914] recommends the drying technique used by Rübow [1904] and by Erlandsen [1907] in which the pulped tissue is dried in thin layers in a current of air at room temperature or at 28°–32°. After drying for 10 hours the material is finely powdered in a mill and the drying finished *in vacuo* over sulphuric acid for 48 hours. This process is very tedious, but we have found it gives as a rule a colourless or pale yellow extract. Other workers have dried the tissue pulp by mixing with anhydrous sulphate of soda [Fränckel and Elfer, 1910] or with plaster of Paris [Windaus, 1910; Rosenheim, 1906; Gardner and various co-workers]. When the mixture has set to a solid mass it is powdered and extracted. The ether extract obtained in this way is but slightly coloured.

Extraction of the ether-soluble constituents.

Thaysen [1914] has investigated the successive extraction of various tissues and fluids, dried by his method, by ether, alcohol-ether, and hydrolysis by soda and extraction by ether. The powdered dry organ was first extracted with ether in a Soxhlet for 48 hours, then exhausted with alcohol in a beaker at 60°–70° for 5–6 hours and again extracted with ether in a Soxhlet. The alcohol extract was worked up with the second ether extract. Finally the extracted powder was placed in a flask with 25 cc. of 20 % aqueous caustic soda and heated in a water-bath for 3–4 hours; then it was poured into a dish, neutralised with hydrochloric acid and evaporated to dryness. The residue was then extracted with ether. In the three ether extracts thus obtained the quantities of free and bound cholesterol were estimated by Windaus' digitonin method.

It is clear from his results, making ample allowance for errors of experiment in the estimation of fractions of milligrams, that both simple ether and alcohol-ether extraction give very imperfect results for air-dried serum. In the case of serum dried by sulphate of soda he obtained better results by ether extraction but they were still too low. He concludes from his investigations that cholesterol and cholesterol esters are bound in the serum in another manner than in the organs, and that in the "serum" itself they circulate in a more or less loose combination with other material. It has been suggested, though without much real proof, that the proteins combine with the sterols.

Bang, for instance [1911], states that the cholesterol esters in a peritoneal fluid are chemically bound to euglobulin.

Thaysen generally concludes that alcohol-ether extraction is a good reliable method; but even simple ether extraction gives very useable results in the case of tissues.

It can be seen from Thaysen's results that simple 48 hours' ether extraction takes out in the case of two samples of liver 99.07 and 97.3 % of the total sterol: in kidney 99.6 and 98.2 %: in suprarenals 99.16 %: and in heart muscle 98.2 and 95.2 % of the total.

In their earlier papers Gardner and Dorée, Ellis and Lander relied on getting the whole of the sterols from tissue dried with calcium sulphate by prolonged ether extraction, usually 10–14 days. This assumption was probably justified, since their values for various tissues are of much the same order as those obtained by Thaysen himself and by later workers by other methods. Lapworth also [1911] showed that drying with calcium sulphate or sulphate of soda did not lead to any appreciable esterification of the free cholesterol. J. Fex [1920] conclusively showed that ether extraction of liver tissue, dried with sulphate of soda, gave quite correct results.

To get over the difficulty of imperfect extraction of dried serum or dried tissue two methods have been used by various observers: (1) use of several solvents, (2) destruction of protein matter by alkalis prior to extraction with ether. Kumagawa and Suto [1908] worked out the relative efficiency of various solvents in extracting fat from heat dried tissues.

Rosenfeld [1900] introduced a method for extracting fatty matter from tissues, which has been much used, and which he claimed gave good results. The tissue is dried with alcohol and heated on a water-bath in a beaker covered by a watch-glass and containing alcohol. This appears to break up any combination, chemical or physical, of the fat with protein. After pouring off the alcohol the residue is extracted in a Soxhlet with chloroform for 4 to 6 hours. The treatment with alcohol and subsequent extraction with chloroform is again repeated. The extracts got by evaporating the alcohol and chloroform solutions separately to dryness are taken up in ether, filtered and the ether evaporated.

The writers have obtained satisfactory extraction by drying the tissue in alcohol, then extracting in a Soxhlet first with alcohol for 24 hours or more and finally with ether. Care must be taken to avoid rubber or cork stoppers. The extracts are evaporated to dryness and taken up in either ether or light petroleum. In our experience a perfect extraction is attained by this method, but the ether extract finally obtained is apt to be dark in colour and sometimes yields a coloured digitonin precipitate in the subsequent sterol estimation.

Other workers destroy the protein by heating with alkali before extracting the sterols. In 1908 Kumagawa and Suto worked out their well-known method for the estimation of fat and unsaponifiable matter in tissues and blood. The method was scarcely new, as a similar procedure had been previously proposed by Liebermann and Székely [1898].

The minced organ is boiled 2 hours with 20 % caustic soda. The solution is placed in a separator, cooled and 20 % hydrochloric acid added in excess, whereby a copious precipitation takes place. Plenty of ether is now poured in and the whole well shaken. After this has stood for some time the ether separates from the other fluid and the precipitate forms a thin layer under the ether. The undermost clear fluid is drawn off, and the upper ether poured off. The precipitate left in the separator is then dissolved in caustic soda and the alkaline solution shaken out with a fresh portion of ether several times. The various ether extracts are combined, evaporated and the residue dried. It is then dissolved in light petroleum. This solution contains the liberated fatty acids and unsaponifiable matter. To get rid of the fatty acids the petroleum solution is brought into a separator and absolute alcohol containing 1 % of potash is added. Water is now added and on standing two layers separate, the soaps in the alcohol layer and the sterols in the light petroleum layer. The advantages of the method are that we get a quantitative extraction, *provided the extraction with ether is repeated a sufficient number of times*. The total cholesterol is in a form suitable for digitonin estimation, and *fresh* tissue or serum can be used without drying.

Since this method was published a large number of methods have been described for estimating cholesterol by colour reactions. These methods have mostly been applied to blood serum. The methods are generally similar, mostly differing in details of extraction, and give fairly similar results. The errors in colorimetric methods have been fully dealt with in Part I, and have also been discussed by Fex [1920]. We only concern ourselves here with the extraction.

Grigaut [1913] mixes 20 cc. serum with 20 cc. caustic soda of 36° Be (i.e. 400 g. per litre) and heats in an autoclave at 110° for one hour. In the case of tissues 5 to 10 g. of the fresh tissue with 40 cc. alkali diluted by half to correspond with the serum conditions are treated similarly. The fluid obtained is shaken in a separator with 60 cc. of ether at 40°–45°. Six successive shakings suffice. When the layers have separated, the lower is drawn into a flask and after heating to 40°–45° is again extracted. The ether is then evaporated and leaves a residue containing cholesterol and some impurities. In order to remove these the residue is dissolved in 50 cc. of 95 % alcohol with addition of 1 cc. of alcoholic soda (1 in 100). He then evaporates to dryness on a water-bath and takes up with light petroleum. On standing, impurities subside and can be easily filtered. The petroleum solution is allowed to evaporate spontaneously and the cholesterol separates in needles and can be weighed after drying at 100°. According to Grigaut, this gives the total cholesterol free and ester. In his colorimetric method 2 cc. of serum are placed in a special cholesterimeter, then 13 cc. of alcohol of 60° containing 1 in 200 of caustic soda. After mixing he adds 15 cc. ether, stoppers the instrument and shakes. After standing he runs off the aqueous layer and replaces by 20 cc. of water. After shaking and standing 5 minutes he again runs off the water and washes with another 20 cc. After removing the water the ether is evaporated in a

porcelain dish. The residue is taken up in 5 cc. of chloroform for the colour test. According to Grigaut, the alcohol by its simple presence is capable of assuring the total passage of the cholesterol into the ether.

Autenrieth and Funk [1913] digest the measured serum on a water-bath for 2 hours with 10 volumes of 25 % caustic potash. The fluid is then extracted with twice its volume of chloroform by shaking for 5 minutes. The chloroform is then separated and the extraction repeated four times. The chloroform extracts are finally dried with sulphate of soda.

Weston and Kent [1917] mix serum with 10 volumes of 95 % alcohol and warm at 60° for 24 hours. The alcohol is filtered and the residue boiled with alcohol. It is then digested 24 hours with ether. The combined extracts are boiled with a few grams of caustic potash for 2 hours, saturated calcium chloride solution added and the precipitate collected on a filter. This is washed with lime-water and extracted with chloroform.

Czonka [1916] takes 2 cc. serum, 20 cc. alcohol and caustic potash and 2 cc. 10 % barium chloride. This mixture is boiled under a reflux for one hour, and after cooling extracted with ether. The ethereal extract is washed with water, evaporated, and the residue taken up in chloroform.

Fex [1920] digests fresh tissue, or blood or serum with excess of 2 % caustic soda solution until the mass becomes gelatinous and goes partly into solution. He then heats on a water-bath for $\frac{1}{4}$ to $\frac{1}{2}$ hour until nearly everything dissolves. The alkaline fluid is then repeatedly extracted with ether.

Bloor [1916] pours the blood or serum in a thin stream into a mixture of three parts of alcohol to one of ether, gently shaken. The mixture is then placed in warm water until the ether begins to boil. It is then cooled and made up to known volume with the alcohol ether mixture.

In processes involving the use of alkali more or less hydrolysis of sterol esters by the alkali takes place, the amount depending on the strength of alkali, temperature and time.

In the colorimetric methods it is assumed that cholesterol and cholesterol esters give the colour reactions with acetic anhydride and sulphuric acid in the same manner, so that it is of no importance whether any hydrolysis is brought about by the alkali or not; the result is the total cholesterol free and combined. That this assumption is not justified, has been pointed out by Gardner and Williams [1921] who showed that in the Liebermann reaction the colour-changes develop at different rates in the case of free sterols and esters, and that in either case variation in the conditions under which the reaction develops has a marked effect on the result.

It is well known that both the gravimetric digitonin method and the colour methods based on Liebermann's reaction (when properly done) give accurate results when pure cholesterol is used. When applied, however, to tissue extracts the two processes often give discordant results. The digitonin process gives very accurately the free cholesterol, and the cholesterol liberated by hydrolysis of esters, *but in the latter case the value of the figure is conditioned by*

the efficiency of the hydrolytic process used. Thaysen, from experiments on cholesterol oleate, maintains that the esters should be boiled for at least 8 hours, and preferably 12, with an alcoholic solution of sodium ethoxide. He used an ester solution in alcohol of about 1.8 %, but did not make it clear what quantity of sodium he used.

We have been in the habit of estimating total cholesterol in an extract by dissolving in ether and adding 10–20 times the theoretical quantity of sodium *ethoxide* in alcohol. As a rule under these conditions hydrolysis is complete on standing overnight in the cold. This procedure has the advantage that less coloured resinous matter is produced than by boiling with alcoholic sodium ethoxide. The ether washings from the cholesterol digitonide should, however, always be tested with Liebermann's reagent to make sure that the hydrolysis was complete. Mueller [1916] has shown that on the same serum Bloor's method gives higher results than that of Autenrieth and Funk, and the latter higher values than the digitonin method. Fex [1920] made an extensive series of estimations of known cholesterol and cholesterol ester solutions by Autenrieth and Funk's method and got results varying in rather wide limits about the mean: he also discussed at length the Grigaut, Bloor and other methods and came to the conclusion that if we wish to attain a certain knowledge of the actual absolute quantities of cholesterol, free and combined, which are contained in blood and tissues, these methods cannot be used. We consider, however, his conclusion too sweeping. We think that there can be no doubt, from a careful survey of different workers' results, that errors due not only to deficient extraction, but to incomplete hydrolysis of esters account very largely for the many conflicting results.

HYDROLYSIS OF CHOLESTEROL ESTERS.

It seemed to us, therefore, that an investigation of the hydrolysis of esters, likely to occur in animal tissues and fluids under various conditions, would enable us to form some estimate of the errors that might arise through inadequate hydrolysis, and possibly to get some criterion of the relative values of the data accumulated by different observers.

For this purpose we selected the three esters which commonly occur and form the bulk of the sterol esters of animal tissues—cholesterol oleate, palmitate and stearate. We also investigated cholesteryl arachidate, which is stated to occur in tissue, and cholesteryl cerotate as an example of an ester difficult to hydrolyse. The esters were in every case tested with digitonin to prove the absence of any free cholesterol. Hydrolysis was investigated under the following conditions: (1) the ester was soaked overnight in 2 % caustic soda solution, and then heated for one hour on a water-bath, as in the method of Fex for treating tissues; (2) heated for 4 hours with 25 % aqueous caustic soda sometimes on the water-bath, but generally in a bath of sulphuric acid so that the fluid actually boiled and kept the difficultly soluble esters well mixed with the alkali; (3) boiled for 4 hours with *N* alcoholic soda; (4) the ester dissolved in

ether, saturated alcoholic sodium ethoxide added and allowed to stand in the cold for 24 hours, conditions used by us in the hydrolysis of tissue extracts.

A very large excess of alkali was used and the quantity of alkali was kept approximately the same per gram of ester. Further the quantities taken for analysis were of the order that would be dealt with in ordinary analyses of tissues. In the case of (1) and (2) the alkaline fluid, diluted if necessary, was thoroughly extracted with ether. In (3) the alcoholic solution after hydrolysis was largely diluted with water and thoroughly extracted with ether. The ethereal solution was then well washed with water. In (4) the hydrolysate was diluted with ether and shaken repeatedly with water to get rid of excess of alkali, soap, alcohol, etc. The wash waters were collected and shaken with ether. The combined ethereal solutions were then again washed with water, evaporated to dryness, taken up in alcohol and precipitated with excess of digitonin. The estimations were made according to the procedure of Fraser and Gardner [1910]. The results are given in the Table, p. 1065.

Cholesteryl oleate was prepared by heating cholesterol with excess of oleic acid to 200° , according to the method of Hürthle [1895]. It melted at 41° – 42° , and had a specific rotary power $[\alpha]_D - 18.5^{\circ}$. A test with digitonin proved it quite free from uncombined cholesterol.

Cholesteryl palmitate was prepared by the method of Abderhalden and Kautzsch [1910] by the action of palmityl chloride (Kahlbaum) in chloroform solution on dry cholesterol. It melted at 77° – 78° and had a specific rotation in chloroform $[\alpha]_D - 24.2^{\circ}$. Tests showed it to be quite free from uncombined cholesterol.

Cholesterol stearate was obtained in good yield by heating 12 g. cholesterol in chloroform solution with 14 g. stearyl chloride (Kahlbaum). It was purified by repeated crystallisation from alcohol. Abderhalden and Kautzsch, who first prepared it in this manner, gave the melting point as 85° – 90° . They gave no rotation and their combustion figures suggested that their preparation was not pure. Our preparation melted sharply at 81° – 81.5° and had a specific rotation in chloroform $[\alpha]_D - 27.6^{\circ}$. A test showed it free from uncombined cholesterol. It seemed to be somewhat more readily hydrolysable than the palmitate.

Cholesteryl arachidate. We have found no previous record of the preparation of this ester, which we carried out by the method of Abderhalden and Kautzsch. 12 g. of arachidic acid were converted into arachidyl chloride by boiling with 25 g. thionyl chloride. The crude arachidyl chloride was dissolved in chloroform and boiled with the theoretical quantity of dry cholesterol. The ester separated on dilution with methyl alcohol. It was purified by recrystallisation from boiling alcohol, from which it separated in white flakes which bound together on rubbing. It melted at 76° – 79.5° and in chloroform solution gave a specific rotation $[\alpha]_D - 22.2^{\circ}$. 100 cc. ethyl alcohol at the boiling point dissolved 0.335 g. and 100 cc. of cold ether 0.856 g. A digitonin test showed it free from uncombined cholesterol, and an estimation of the cholesterol after hydrolysis showed that it was pure.

Cholesteryl cerotate. We had some difficulty in making this compound as we were unable to purchase pure cerotic acid. According to Marie [1896], cerotic and melissic acids occur together as esters in carnauba wax and Chinese wax and are not easy to separate and purify. Two specimens of commercial acid melted at 66°–67° and 63°–65° respectively. We were unable to ascertain the source from which these specimens were made, but both contained unhydrolysed wax. The crude acid was boiled with an alcoholic solution of sodium ethoxide. The alkaline soap obtained was thoroughly washed with ether and the soap solution extracted with ether. From the ethereal extract we obtained an alcohol melting at 82°–83°, perhaps myricyl alcohol. The acid liberated from the soap by mineral acid was taken up in ether. After evaporation of the ether, the acid was repeatedly recrystallised from alcohol, in which it was very difficultly soluble. The final crop melted at 80°–82° and separated from the alcohol as a white powder. The melting point of cerotic acid is given in the literature as 77.5° and of melissic acid as 90°–90.6°. Melissic is less soluble in methyl alcohol than cerotic acid.

Our acid was probably mainly cerotic with some melissic acid. For our purpose this did not much matter, since our object was to ascertain whether esters of such acids were more difficultly hydrolysable than those of palmitic, stearic, etc. 6.8 g. cerotic acid were converted into chloride by means of thionyl chloride, and the crude cerotyl chloride taken up in chloroform and boiled with 7 g. dry cholesterol. The ester separated on pouring into alcohol. It was purified by repeated recrystallisation from a large volume of boiling alcohol in which it was only sparingly soluble. It separated as a white crystalline powder, which on heating began to soften at 65° and melted at 73°–75°. In chloroform solution it had a specific rotation $[\alpha]_D - 5.3^\circ$ approximately. 100 cc. of boiling ethyl alcohol dissolved 0.296 g. A test with digitonin showed that it contained a minute trace of cholesterol—0.0004 g. in 0.1857 g., i.e. about 0.2 %.

The results of the hydrolysis of these esters under the conditions mentioned above are summarised in the following Table:

Ester	2 % caustic soda solution	Heated 4 hours with 10 cc. of 25 % aqueous caustic soda			Boiled 4 hours with 60 c.c. of N alcoholic soda			Ether solution of ester allowed to stand 24 hrs. in cold with alcoholic sodium ethoxide		
		wt. of ester, g.	wt. of digitonin comp., g.	% hydro- lysis	wt. of ester, g.	wt. of chol. digi- tonide, g.	% hydro- lysis	wt. of ester, g.	wt. of digitonin comp., g.	% hydro- lysis
Oleate ¹	No action	0.0516	0.1283	100	0.0493	0.1340*	100	0.0504	0.1266*	100
Palmitate	"	0.0582	0.0582	39	0.0621	0.1633	100	0.0488	1.1364	100
		0.0510	0.0301	23						
Stearate	"	0.0481	0.1124	95–96	0.0520	0.1353*	100	0.0497	0.1180	98
Arachidate	"	0.0438	0.0915	89	0.0521	0.1248	100	0.0391	0.091	100
		0.0514	0.1060	88–89						
Cerotate ²	"	0.0371	0.0346	45	0.0566	0.0433	65	0.1147	0.2004	84

* These digitonides were slightly coloured by resinous matter.

¹ In an experiment in which cholesteryl oleate was allowed to stand 24 hours in the cold with alcoholic sodium ethoxide, without the addition of ether, hydrolysis was incomplete; 0.0368 g. of oleate gave only 0.0612 g. of compound, corresponding to 68 % hydrolysis. This was no doubt due to the sparing solubility of the ester in cold alcohol, for even at the boiling point 100 cc. of alcohol only dissolve 0.4 g. of the ester.

² The cerotate when refluxed in ether solution with sodium ethoxide underwent 86 % of hydrolysis and was completely hydrolysed by 4 hours' boiling with alcoholic sodium ethoxide.

We consider that these experiments afford an experimental justification of the method adopted by one of us and various co-workers for the hydrolysis of esters in tissue analysis, viz. treatment of an ethereal solution with excess of alcoholic sodium ethoxide in the cold.

They also afford some explanation of the varying results obtained in comparative experiments by the methods of Bloor, Autenrieth and Funk, and Grigaut, since, as has been shown by Gardner and Williams [1921], the cholesterol esters develop colour with the Liebermann reagent at quite different rates and in some cases with different tints from pure cholesterol. The colour reaction methods when applied to tissues are also rendered less precise owing to the substances of non-sterol character which accompany cholesterol and its esters in the tissue extracts and which give more or less brown coloration with acetic anhydride and sulphuric acid and modify the green tint due to the cholesterol so that comparison with the pure green tint of the standard is difficult. This is also accentuated as we have previously shown by the production of traces of resinous bodies during hydrolysis which also give yellow-brown or greenish colours with the Liebermann reagent. The colour methods also have the disadvantage of being micro methods, so that errors of experiment inherent in the most skilful work become enormously magnified when results are calculated to percentages. These hydrolysis experiments also threw light on some of the results obtained by one of us [Gardner, 1921] on the sterols of faeces. In that paper it was shown that after removing free sterols by digitonin, hydrolysing the esters by means of the action of a large excess of sodium ethoxide in an ethereal solution of the material and again removing the sterols, thus set free, by means of digitonin, a residual oil could be obtained which was capable of being distilled in superheated steam or in a high vacuum without decomposition. The purified oil thus obtained gave as a rule a brown colour in chloroform solution with acetic anhydride and sulphuric acid, but in some cases the brown gradually changed to a dusky green. The latter oils on hydrolysis in sealed tubes with alcoholic caustic soda in large excess yielded traces of sterols again precipitable by digitonin. Evidently the faecal waxes contained small quantities of stable esters such as cholesterol cerotate, which escaped hydrolysis by sodium ethoxide in ethereal solution. Possibly such esters may occur also in minute quantity in tissues and body fluids, and in order to insure complete hydrolysis of an ether extract it is perhaps advisable to boil in alcoholic solution with sodium ethoxide for several hours.

Since the cholesterol esters that occur in tissues are quite unaffected by a 2 % solution of caustic soda, Fex's method of extraction presents distinct advantages. His procedure is as follows. The required weighed quantity of minced fresh tissue is mixed with about twice its weight or more of 2 % caustic soda. It is allowed to digest at room temperature for several hours until the tissue swells up and a gelatinous transparent mass is obtained. The flask is then put on a boiling water-bath until the gelatinous stuff goes largely into solution. This generally takes about half an hour. A brownish-red

moderately clear liquid is obtained. This is put into a separator and thoroughly shaken with ether, and allowed to stand at least 12 hours to insure complete separation of the ethereal and aqueous layers. This extraction is again repeated with a fresh lot of ether. We have generally found that more than two extractions are necessary. The ethereal solutions are combined, the ether removed by distillation and the residue dried, preferably in our experience *in vacuo*.

This process is easier and quicker than the sulphate of soda or calcium sulphate processes, or than the alcohol-ether processes. Fex found that for liver and kidney at any rate his process gave results agreeing with the sulphate of soda ether extraction process. He also found identical values, within errors of experiment, in parallel estimations, using this process and alcohol extraction according to the procedure of Schimidzu.

We are able to confirm fully Fex's claims as to the advantage of this method in tissue analysis, and that it avoids the displacement of the free to ester ratio which is sometimes noticed in tissues that have been air-dried or treated by the alcohol-ether process. We think its most important advantage is that the fresh tissues can be treated at once, so that possible autolytic or bacterial changes that may take place on standing for drying purposes are obviated.

Recently one of us has applied Fex's process to the analysis of blood plasma with very satisfactory results. When extracted by Bloor's process, or by drying with alcohol and subsequent alcohol and ether extraction, we have noticed occasionally that in the estimation by digitonin very troublesome colloidal suspensions were formed in washing the cholesterol digitonide, which made filtration difficult. This has not occurred in the extracts by Fex's process.

Inorganic esters of cholesterol.

In 1898 O. Hammarsten examined the bile of the great shark (*Scymnus borealis*) and found that it contained no cholesterol, and neither bilirubin nor urobilin [1898]. The mixture of sodium salts obtained by precipitating the bile acids with lead acetate and decomposing the precipitate with sodium carbonate, though responding strikingly to Pettenkofer's reaction, and the fluorescence test, contained no glycocholic or taurocholic acid. It consisted instead of the salts of at least two acids containing sulphur, which differed from taurocholic acid in losing the whole of their sulphur in the form of sulphuric acid in hydrolysis by acids. He named the two chief acids α - and β -scymnolsulphuric acid. The soluble α -scymnolsulphate was precipitated from a 3 % aqueous solution of the mixed salts by adding an equal volume of 40 % aqueous potash. The substance was white, containing 5.346 % of sulphur and produced a characteristic indigo blue colour when dissolved in 25 % hydrochloric acid: it was easily soluble in ethyl and methyl alcohol. By hydrolysis he isolated a crystalline substance, melting at 100°–101°, α -scymnol. This gave Pettenkofer's reaction, and the fluorescence test for cholic acid, and the same colour reaction with 25 % hydrochloric acid as the sulphate.

It resembled cholesterol in giving Schiff's test and the Borchard-Liebermann reaction with acetic anhydride and sulphuric acid.

He also described β -scymnol which he regarded as the next higher homologue of α -scymnol. He did not apparently isolate the substance in a sufficiently pure state to make his analysis by combustion of much value. He also obtained indication of the presence of such ethereal sulphates in human bile and in that of suckling animals. Probably Hammarsten had in hand some paired compound of cholesteryl ethereal sulphate and bile acid.

This led Mandel and Neuberg [1915] to prepare and examine ethereal sulphates of cholesterol. They succeeded in preparing cholesteryl potassium, sodium and ammonium sulphates by acting with chlorosulphonic acid on dry cholesterol, and neutralising the acid with the appropriate alkali.

We prepared both salts, following closely Mandel and Neuberg's directions. The salts were purified by repeated crystallisation from 80 % alcohol.

The sodium salt proved considerably more soluble in water than the potassium salt, and the ammonium salt was still more soluble. Mandel and Neuberg gave the melting point of their potassium salt as 235° with decomposition. Our preparation melted lower than this at 181° – 183° with decomposition. They gave the melting point of the sodium salt as 163° – 164° with decomposition. Our preparation melted at 163° – 165° , with decomposition.

Sodium cholesteryl sulphate was practically unaffected by boiling with aqueous alkalis, and even with sodium ethoxide. It was, however, readily hydrolysed by acids. 0.0492 g. was boiled under a reflux for 4 hours with glacial acetic acid. The solution was then diluted with water and extracted with ether. The ethereal solution was freed from acid by washing with water and then with dilute alkali and evaporated to dryness. The residue, consisting of cholesterol and cholesterol acetate, was hydrolysed in the usual way with sodium ethoxide, diluted and extracted with ether. 0.1587 g. cholesteride was obtained corresponding to 0.0386 g. cholesterol. Hydrolysis was complete and the salt pure.

Potassium cholesteryl sulphate. This was also unacted on by aqueous alkalis. 0.0474 g. treated with acetic acid in the above manner yielded with digitonin 0.1486 g. of compound, equivalent to 0.0361 g. cholesterol; hydrolysis complete.

It is evident from these results that any cholesterol in the form of an alkali ethereal sulphate would be missed in any of the usual processes of extraction. Such cholesterol could only be detected and estimated by taking the material that had been extracted in the ordinary manner and boiling it with acid—say glacial acetic—for several hours. The acetic acid may then be distilled off, or neutralised and the fluid left extracted thoroughly with ether. The cholesterol extracted in this manner may, after hydrolysis of any ester of acetic acid, be estimated in the usual way. It is not excluded that cholesterol found in this manner might have been present in some other compound than an ethereal sulphate—for example, as an ethereal phosphate, which presum-

ably might have properties similar to an ethereal sulphate. As far as we are aware, however, no such phosphates have yet been described.

One of us has found traces of such cholesterol in the bile of the hippopotamus [Gardner, 1924]. We have also detected it in some specimens of human bile but not all, and also in normal human urine. An account of this work, however, we hope to make the subject of a future communication.

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