XC. STUDIES ON THE CHEMICAL NATURE OF THE UNSAPONIFIABLE FRACTION OF FISH OIL.

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SINCE it was shown [Steenbock and Boutwell, 1920] that both vitamin A and the antirachitic vitamin [Zucker, Pappenheim and Barnet, 1922] in fish oils after saponifying are found in the unsaponifiable fraction, there has been no lack of attempts to prepare the active substances in a pure state. The results have hitherto been negative [see especially Drummond, Channon and Coward, 1925]. The isolation of the high molecular alcohols and hydrocarbons present in the fraction is rendered difficult by the circumstance that as a rule it is necessary to work with small quantities. It has not been possible to produce characteristic derivatives from which the original substances could easily be regenerated. Fractional distillation in a vacuum has been attempted. This method, however, is of little use in isolating substances which occur in complicated mixtures and in small quantities. A number of distillations which the present writer carried out at 0.01–0.02 mm. mercury pressure gave fractions which in some cases were very active, but which were not sufficiently well defined to give encouragement for continued research.

The object of the investigations of which a preliminary report will be given below was to convert the alcohols present into acid esters by the aid of the anhydrides of bivalent acids. I hoped to obtain a satisfactory separation by means of the solubility-properties of the free ester acids or their salts.

As a readily accessible material for commencing the experiments was selected the unsaponifiable fraction of liver oil of the Greenland shark, *Somniosus microcephalus*—a species of shark commonly found off the northern coasts of Norway. This oil is obtainable in sufficient quantities, for it is an object of industrial production. It contains 10–15 % of unsaponifiable substance, so that considerable quantities of the latter can be obtained in an ordinary laboratory without difficulty. The content of vitamin A of the oil is about the same as that of ordinary good cod-liver oil, since in daily doses of 2 mg. it produces growth in rats, whose growth has been checked as the result of lack of vitamin A.

As cod-liver oil contains about 0.5 % of unsaponifiable substance, the concentration of the active substances in the unsaponifiable fraction of

Somniosus oil is only one-twentieth to one-thirtieth as great as the vitamin concentration in the corresponding fraction of cod-liver oil.

The oil of *Somniosus microcephalus* was previously examined by Tsujimoto and Toyama [1922], who isolated, besides cholesterol, the two hitherto unknown alcohols, viz. batyl alcohol, $C_{20}H_{42}O_3$, and selachyl alcohol, $C_{20}H_{40}O_3$, both dihydric.

Preparation of the unsaponifiable fraction of Somniosus oil.

After saponifying with an alcoholic solution of potassium hydroxide in excess, the bulk of the fatty acids are precipitated as Ca-soaps by the addition of an alcoholic solution of $CaCl_2$; simultaneously the excess of KOH is precipitated in accordance with the equation

In this way an almost neutral filtrate is obtained, which, in addition to unsaponifiable substance and glycerol, still contains Ca-soaps soluble in alcohol. These are removed, and the glycerides that may still be present are saponified, by re-heating with a moderate excess of alcoholic potassium hydroxide. The Ca-soaps are thereby converted into K-soaps, soluble in water, whilst the calcium hydroxide is precipitated. The filtrate is evaporated in a vacuum and dissolved in ether. The K-soaps and the glycerol are removed from the ethereal solution by repeated washing with water.

The yield was fairly good, and as a rule amounted to 9–10 % of a brownish-yellow, half-solid mass, which contained about 14 % of cholesterol (determined colorimetrically by Bloor's method). Somewhat better yields were obtained by leaving out the precipitation by $CaCl_2$ (the voluminous Ca-soaps adsorb a great deal of ether-soluble substance, which is only washed out with great difficulty), and after adding water shaking out the alcoholic solution of the K-soaps with ether. Large quantities of ether are required for this, and now and then unpleasant emulsions are formed.

Separation of cholesterol and batyl alcohol.

170 g. of unsaponifiable substance are dissolved by heating in 225 cc. of absolute alcohol. On cooling down to 0° there are obtained after a considerable time 22 g. of a firm, white substance, consisting of a mixture of cholesterol and batyl alcohol. These are separated by means of fractional crystallisation from alcohol in which cholesterol is less soluble. The batyl alcohol is freed from cholesterol by precipitation with digitonin by Windaus' method, is transferred to ether, and the excess of digitonin is removed by shaking with water. Finally, the batyl alcohol is recrystallised five times from absolute alcohol. The preparation thus obtained was white, finely crystallised and melted at 69° . The analysis also agreed with the figures of Tsujimoto and Toyama:

7.899 mg. gave 21.158 mg. CO_2 and 8.942 mg. H: 73.00 % C and 12.67 % H.
Calculated for $C_{20}H_{42}O_3$: 72.65 % C and 12.79 % H.

A methoxyl determination showed that the batyl alcohol contains one OCH_3 group:

8.009 mg. gave 5.878 AgJ: 9.69 % OCH_3 .

Calculated for $\text{C}_{19}\text{H}_{37}(\text{OH})_2(\text{OCH}_3)$: 9.39 % OCH_3 .

The pure batyl alcohol had neither growth-promoting nor antirachitic properties.

The liquid part of the unsaponifiable fraction.

After removing the cholesterol and batyl alcohol, the alcoholic mother liquor was evaporated in a vacuum. The residue was a clear, reddish-brown, readily flowing oil. It was dissolved in acetone and kept at 5° for several days, during which time a small amount of solid substance separated which melted even at room temperature and was not further examined for the time being.

After evaporating the acetone, the remaining oil still contained 8 % of cholesterol. After this had been removed by precipitation with digitonin, the acetyl number of the fraction was 254.4. It was found, however, by saponification that it still contained about 6 % of glycerides. Taking this into consideration, we obtain for the alcohols present a calculated acetyl number of 270.6 (selachyl alcohol 272.1). The iodine number of the fraction (Hübl's method, standing for 8 hours) was 87.5; calculated for selachyl alcohol 79.2.

The fraction thus appears to consist principally of selachyl alcohol.

Preparation of the acid phthalic esters.

Drummond, Channon and Coward failed in attempts to produce acid phthalic esters of the higher alcohols of cod-liver oil by the method used by Willstätter and Stoll [1913] when preparing the acid phthalic ester of phytol by boiling with phthalic acid anhydride in a benzene solution.

The acid phthalic esters are obtained in good yield by treating the alcohols with melted phthalic anhydride, or by boiling a solution of the alcohols and phthalic anhydride in pyridine for a short time. A considerable quantity of dark coloured substances is formed during these operations. This could be avoided to a great extent by allowing the mixture to remain at the ordinary temperature. It is easy to follow the reaction by taking samples from time to time, and after adding water titrating the free phthalic acid with potassium hydroxide to phenolphthalein. The pyridine present has no influence on the result. By using about 100 % excess of phthalic anhydride, after standing for 3 days at a temperature of 20° , ca. 90 % of the estimated quantity of phthalic anhydride was used.

To 0.5195 g. of alcohols are added 5 cc. of a solution of phthalic anhydride in freshly distilled pyridine, which, after adding water, required for neutralisation 38.2 cc. 0.3304 *N* NaOH, corresponding to 0.9338 g. of phthalic anhydride. After standing for 3 days at 20° , 29.38 cc. are required for neutralisation, i.e. the carboxyl groups corresponding to $38.20 - 29.38 = 8.82$ cc. of 0.3304 *N* NaOH have been esterified. This corresponds to a consumption of phthalic anhydride of $2 \times 0.074 \times 0.3304 \times 8.82 = 0.4313$ g. For 0.5198 g. selachyl alcohol, $\frac{148 \times 2 \times 0.5198}{328} = 0.4689$ g. is required. Thus 92 % of the calculated quantity of phthalic anhydride is bound.

The diphthalic ester acids are suitable for fractionating the liquid portion of the unsaponifiable substance, as shown by the following experiment: 5 g. of the liquid part of the unsaponifiable fraction were dissolved in 25 cc. of pyridine, 10 g. of phthalic anhydride were added, and the solution boiled for 1 hour under reflux, after which the solution was poured into 400 cc. of warm water. By the addition of some more pyridine almost all was dissolved. After cooling the solution was filtered and the filtrate was washed with 250 cc. of ether. The ester acids passed into the ether as pyridine salts. (A sample of the solution gave no precipitate on the addition of H_2SO_4 in excess.) The ethereal solution of pyridine salts was repeatedly washed in dilute H_2SO_4 and then once or twice with water. The ether solution, which was reddish-brown, was dried with Na_2SO_4 , filtered and evaporated, yielding a residue of 7.34 g. The equivalent weight of the preparation was found to be 383. Calculated for the selachyl monophthalic ester acid 478; for the selachyl diphthalic ester acid 311.

Thus there was probably present a mixture of mono- and di-phthalic ester acids, and the esterification had by no means been complete.

A portion of this preparation was dissolved in dry ether and several volumes of light petroleum ($30^\circ\text{--}50^\circ$) were added, whereby a considerable quantity of dark-coloured, viscous substance was precipitated. The petroleum layer, which after standing became quite clear, left after evaporation 1.569 g. of brown viscous oil, the equivalent weight of which was 507. The dark, viscous, precipitated substance was shaken up two or three times with light petroleum, dissolved in ether, and the ether evaporated. The weight of the residue was 3.155 g. and its equivalent weight 341. It thus appeared that it is possible to separate mono- and di-phthalic ester acids by the aid of their different solubilities in light petroleum. Thus even if complete esterification is not achieved it will be possible to arrive at pure preparations of diphthalic ester acids by precipitating with light petroleum. The solution in light petroleum will, in addition to monophthalic ester acids, contain any hydrocarbons, glycerides and cholesterol phthalic ester acids which might be present.

If we wish to isolate the hydrocarbons, we may remove the ester acids from the light petroleum solution by washing with alkali, or we can dissolve the fraction in alcohol and precipitate the phthalic ester acids as Pb salts by the addition of an alcoholic solution of Pb acetate. The oil of *Somniosus microcephalus* contained very little hydrocarbon, since only 70 mg. could be isolated from 20 g. liquid unsaponifiable substance. After the bulk of the cholesterol had been removed by crystallisation from an alcoholic solution, the unsaponifiable substance of cod-liver oil contained about 9 % of hydrocarbons.

The acid phthalic ester of cholesterol is prepared by boiling a solution of 1 g. of cholesterol and 2 g. of phthalic anhydride in 5 cc. of pyridine for 1 hour. The mixture is poured into water and the precipitated ester acid is removed by filtration. In contrast to the selachyl diphthalic ester acids, the pyridine

salt does not appear to exist in a watery solution. The precipitate is treated with dilute HCl and recrystallised twice from alcohol, whereby white crystals are obtained, which melt at 161–161.5°. The equivalent weight was 539, calculated 534. The cholesterol phthalic ester acid is easily soluble in ether, but is not precipitated by light petroleum, even when considerable quantities of the latter are added. In this way it is possible to free our preparations from cholesterol without resorting to digitonin, the high price of which renders its use difficult in sufficiently large quantities.

Batyl diphthalic ester acid. 2 g. of batyl alcohol and 4 g. of phthalic anhydride, dissolved in 25 cc. of pyridine, were allowed to stand for 2½ days at room temperature. After diluting with water the solution was washed repeatedly with ether. After washing with dilute sulphuric acid and finally with water, the ethereal solution was dried over Na_2SO_4 , filtered and evaporated. The residue was 3.332 g. of a firm, white substance, which melted at 91°. It was dissolved in ether and precipitated with light petroleum. After this had been repeated twice, the melting point was constant at 97°. The equivalent weight was 312.2; calculated for $\text{C}_{20}\text{H}_{40}\text{O}_3(\text{C}_8\text{H}_5\text{O}_3)_2$, 312.

Preparation of selachyl alcohol by the aid of salts of the diphthalic ester acid.

We did not succeed in producing selachyl diphthalic ester acid in crystalline form. It always forms jellies when separating from its solutions.

Attempts were therefore made to use various salts for preparation of the pure phthalic ester acid. Lead and copper salts appeared to be best suited for this purpose. The potassium, sodium and magnesium salts are soluble in water.

The lead salt of selachyl diphthalic ester acid. 3 g. of potassium salt were dissolved in 25 cc. of water and precipitated with 3 g. of neutral lead acetate, which also was dissolved in 25 cc. of water. The precipitate, after being dried over sulphuric acid in a vacuum desiccator, weighed 3.2 g. It was almost completely soluble in ether and was again precipitated by adding alcohol. After twice precipitating in this way, the analysis gave the following results:

0.2292 g. salt gave 0.0856 g. PbSO_4 . Found 25.51 % Pb, calculated 24.91 % Pb.

The lead salt was decomposed by shaking an ethereal solution with dilute hydrochloric acid and then with water. The phthalic ester acid thus isolated had the iodine number 42.4; calculated 40.7. If the conditions were altered, a Pb salt of another composition was precipitated. If an alcoholic solution of the potassium salt of the ester acid was precipitated by an alcoholic solution of lead acetate, a precipitate was obtained which, after being thrice dissolved in ether and precipitated by alcohol, showed a constant melting point of 140°. 0.2024 g. of this gave 0.0904 g. of PbSO_4 , corresponding to 30.57 % Pb. From this was produced the free ester acid and from the latter the free alcohol. In a dose of 0.01 mg. daily this was found to produce growth in young rats, the

growth of which had been checked on account of lack of vitamin A. The active substance, however, is evidently only precipitated by alcohol along with the Pb salt. The precipitate was amorphous, and the ethereal solution had colloidal characters. It also appeared (see below) that the preparation of selachyl alcohol obtained from the crystalline copper salt had neither growth-promoting nor antirachitic properties.

Preparation of the copper salt. 16.9 g. of the phthalic ester acid with equivalent weight 320, i.e. fairly pure diphthalic ester acid, were dissolved in 75 cc. of alcohol and neutralised by means of 160 cc. of 0.3304 *N* NaOH. To the solution thus obtained were added 17 g. of crystalline copper sulphate, dissolved in 100 cc. of water. The precipitate thus obtained, which weighed 18 g., was almost completely soluble in 650 cc. of boiling alcohol, and on cooling crystallised out. After recrystallising several times, 0.0986 g. gave 0.0114 g. CuO, corresponding to 9.24 % Cu; calculated for the copper salt of selachyl-diphthalic ester acid, 9.25 % Cu.

The free ester acid is produced by passing H_2S into a boiling alcoholic solution of the copper salt. CuS is precipitated colloiddally, but is brought to an easily filtrable form by shaking with Fuller's earth. The free ester acid is a very viscous, faintly yellowish oil, which showed no signs of crystallisation after standing.

The free alcohol was produced by saponification of the ester acid with alcoholic potash and subsequent solution in ether. The ether was evaporated with water and left a faintly yellowish, readily flowing oil.

7.745 mg. gave 20.647 mg. of CO_2 , and 8.691 mg. of H_2O , corresponding to 72.73 % C and 12.56 % H. Calculated for $C_{20}H_{40}O_3$, 73.07 % C and 12.30 % H.

This preparation of selachyl alcohol showed by animal experiments in daily doses of 0.16 mg. neither growth-promoting nor antirachitic properties, and moreover did not give the well-known colour reaction with concentrated sulphuric acid, either alone or after the addition of cholesterol.

Preliminary remarks regarding the unsaponifiable fraction of cod-liver oil.

It is intended to investigate the unsaponifiable fraction of cod-liver oil on the lines indicated above. Some preliminary experiments have given the results that this fraction (prepared in the same manner as that described for shark oil), after the cholesterol had as far as possible been removed by crystallisation from alcohol, still contained 14.3 % of cholesterol. The acetyl number was 222.3, the iodine number 154.2 and there was about 9 % of hydrocarbons present. Drummond, Channon and Coward found *ca.* 8 % spinacene. Correcting the figures found for acetyl and iodine numbers according to these data, we obtain for the alcohols present an acetyl number of 265 and iodine number 144. The acetyl number of the alcohols should indicate that there are present compounds similar to those in shark oil, but the high iodine number indicates that they must be more unsaturated than selachyl

alcohol. Orienting experiments have further shown that the alcohols of cod-liver oil by esterification with phthalic anhydride in pyridine behaved analogously to selachyl alcohol, forming diester acids, insoluble in light petroleum. Their equivalent weight was 314.

Of the salts, that of barium appeared to be best suited for the preparation of the pure acid, for it was fairly easily soluble in boiling alcohol and separated out on cooling. These investigations are being continued by the present writer.

SUMMARY.

1. The alcohols present in the unsaponifiable fraction of the liver oil of *Somniosus microcephalus* can be converted into phthalic ester acids, which may be used for the isolation of the alcohols.

2. The bulk of the alcohols in the oil of *Somniosus microcephalus*, and presumably also of the alcohols in cod-liver oil, are dihydric.

3. The properties of batyl alcohol and selachyl alcohol produced from the oil of *Somniosus microcephalus* agree with the statements of Tsujimoto and Toyama.

4. In the pure condition these alcohols have neither growth-promoting nor antirachitic properties, and do not give the well-known colour reaction with sulphuric acid.

5. The ester acids of the alcohols which are present in cod-liver oil exhibit a close resemblance to selachyl diphtalic ester acid. The alcohols of cod-liver oil, however, are more unsaturated than selachyl alcohol.

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