LV. THE BIOLOGICAL SIGNIFICANCE OF THE UNSAPONIFIABLE MATTER OF OILS.

II. AN UNIDENTIFIED UNSATURATED HYDROCARBON PRESENT IN MAMMALIAN LIVER.

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Reference has been made in the preceding paper [Channon, 1926] to work which is being carried out on squalene, a hydrocarbon of the formula $C_{30}H_{50}$, described by Tsujimoto [1916, 1920], which appears to be identical with a hydrocarbon, spinacene, isolated independently by Chapman [1917, 1918, 1923], who ascribed to it the formula $C_{29}H_{48}$. In that paper the biological interest of this highly unsaturated hydrocarbon, which is present to the extent of some 80 % of the liver oils of certain sharks, has been briefly discussed. Its occurrence outside the Elasmobranchs has been reported by Drummond, Channon and Coward [1925], who found it present as some 8 % of the unsaponifiable matter of cod-liver oil. Toyama [1923] reports the presence of another hydrocarbon, pristane, in fish-liver oils, and various alcohols of high molecular weight have been described, such as batyl alcohol, $C_{20}H_{42}O_3$, chimyl alcohol, $C_{19}H_{42}O_3$, and selachyl alcohol, $C_{20}H_{40}O_3$ [Tsujimoto, 1922, 1923, 1924]. The occurrence of substances such as these in fish-liver oils made it of interest to see whether they were present in livers other than those of fish, and this paper deals with an investigation into the unsaponifiable matter of the livers of certain animals which was undertaken with a view to proving the presence or otherwise of the unsaturated hydrocarbon, squalene, $C_{30}H_{50}$.

Preparation of the unsaponifiable matter from livers.

The preparation of this material was carried out by the method described in the previous paper. Briefly, the minced liver was heated in 5 % aqueous potassium hydroxide solution until it had dissolved, when the solution was cooled and strained into large separators where it was diluted with several volumes of water. The solution after the addition of alcohol was exhaustively extracted with ether and the combined ether extracts were washed with water

until free from soap, when the solvent was removed. The product so obtained was usually an oil containing much crystalline material (cholesterol) and was dried at 100° *in vacuo*. It was then resaponified with sodium ethylate to ensure the hydrolysis of esters of cholesterol and other higher alcohols possibly present and the solution, after being diluted many times with water, was extracted with ether as before. The ether extracts were then very thoroughly washed with water, and the ether evaporated. The final product was dried at 100° *in vacuo* and the flask sealed by a waxed cork. In this way were treated several kg. of human, pig, sheep, ox, and horse livers. These materials were stored for about 5 weeks with a view to their being worked up together. The livers, save in the first case, were taken from animals immediately after death, and were minced while still warm into potassium hydroxide solution on reception from the slaughter-house. The human material was some 48 hours old. The yields of unsaponifiable matter obtained were between 0·3 and 0·4 %. A similar figure was obtained for rats' livers [Channon, 1926], so that it would appear that in general there is present in liver some 3·5 g. of unsaponifiable matter per kg. wet weight. In the preparation, connective tissue was, as far as possible, excluded. The material so obtained varied somewhat in consistency but had as a rule a hard waxy appearance due to the presence of a large proportion of cholesterol, and was yellow to orange red in colour on account of the presence of the lipochrome pigments carotene and xanthophyll. That from man, ox, and sheep was very similar, deep red in colour and hard; that from horse liver was very much paler but of the same consistency, whilst material from the pig varied in colour considerably from very pale yellow to deep orange red, and appeared to be of a more oily nature although containing a very high proportion of cholesterol. No estimations were made of the cholesterol contents of these materials, but previous work has shown that there is usually present some 70 % of that substance.

Treatment of the unsaponifiable matter. In the previous paper mention has been made of the fact that squalene gives rise to a hexahydrochloride, $C_{30}H_{50}6HCl$, when hydrochloric acid gas is passed into its solution in ether or alcohol. This derivative, which crystallises in hexagonal and diamond-shaped plates, is very characteristic and serves for the detection of the parent substance. Its melting point varies, but after two recrystallisations from acetone a white crystalline substance, M.P. 113°, results. It was decided to make use of the ready formation of this derivative in the search for squalene in these unsaponifiable liver materials. Some little difficulty was encountered at first owing to the large amount of cholesterol present, but a preparation of cholesterol hydrochloride showed that that substance crystallised in long silky needles, M.P. 155°, as stated by Mauthner [1906]. On recrystallisation from hot acetone it forms a gel which will not flow from an inverted tube. As it was hoped to recrystallise squalene hexahydrochloride from acetone, should that substance be found present, it was finally decided, after experiments on mixtures of cholesterol and squalene hydrochlorides, to overcome

this difficulty by removing the cholesterol derivative by grinding up the precipitate in a mortar with ether several times, and subsequently to recrystallise the ether-insoluble residue from acetone. The procedure adopted therefore was to pass dry hydrochloric acid gas into a solution of the unsaponifiable matter in ether and alcohol. The ratio of ether to alcohol was 4 : 1 and the concentration of unsaponifiable matter about 6 %. On passing hydrochloric acid gas into such a solution of the unsaponifiable matter, the colour rapidly changes to dark brown and finally goes quite black, and after about 12 hours a precipitate begins to appear. The solution after cooling on ice is filtered under reduced pressure when there remains a dark mass, amorphous for the most part, but containing also crystalline cholesterol hydrochloride. The latter is removed by grinding the mass in a mortar with ether and filtering and repeating this operation three or four times. The residue on drying is a light brown powder resembling in appearance the bromides of the highly unsaturated liver acids. This is dissolved in boiling acetone and filtered; there appear on cooling fawn-coloured crystals which under the microscope are seen to be very small needles which tend to form stellate clusters. Further recrystallisation does not result in any apparent improvement in colour, and we have been unable to obtain a white product. This substance did not seem to be the hydrochloride of squalene, for the latter can be readily obtained in beautiful white plates by crystallisation from acetone; it melted at 128° and contained about 31 % chlorine, whilst squalene hydrochloride melts at 113° and contains 33·86 % chlorine. The amounts of the substance we were obtaining at that time prevented any very definite evidence being obtained, but analysis made it appear that the substance which was giving rise to the hydrochloride was an unsaturated hydrocarbon other than squalene. This hydrochloride was originally obtained from the unsaponifiable matter of human liver; later an apparently similar substance of the same crystalline form, melting point, and chlorine content was obtained from the unsaponifiable matter of pig, ox, horse, and sheep liver. We thus tended to the belief that there was present in these materials a hydrocarbon which did not appear to be squalene; nevertheless the possibility that it was squalene which was giving rise to the hydrochloride was still kept in mind, for we could not be sure of the purity of such small amounts of material.

Formation of an insoluble bromide. Squalene gives rise to a bromide ($C_{30}H_{50}Br_{12}$) which can be formed by the addition of bromine in ether to an ether solution of squalene. The yield is by no means quantitative, and the reaction appears to be complicated by the formation of secondary products. The yield normally obtained is of the order of 35 %. It seemed reasonable to expect that the substance giving rise to the hydrochloride described above would also form an insoluble product on addition of bromine: this substance was readily obtained. On addition of excess of bromine to a solution of the unsaponifiable matter in ether, a precipitate is immediately formed, and, if this is filtered off, any cholesterol dibromide present in it can be readily

removed by frequent washings with ether. This substance, which is also not quite white, contains about 68 % bromine, is amorphous, and does not melt, but begins to char at 170° and goes black at 180°. Squalene dodecabromide behaves similarly when an attempt is made to melt it, but the latter contains 70·1 % bromine. In every case where a sample of unsaponifiable matter had given a hydrochloride by the method described above, it also gave a bromide on addition of bromine in ether.

It was therefore concluded that the unsaponifiable matter of the livers of man, ox, horse, sheep, and pig contains a hydrocarbon, which gives rise to a hydrochloride and a bromide very similar in halogen content to the corresponding squalene derivatives. The evidence suggested that this substance was not squalene, but no definite opinion was possible at that stage. It was therefore decided to work up a large quantity of material, and the liver of the pig was chosen, because there seemed to be present in the unsaponifiable matter of pig's liver a larger proportion of the unsaturated hydrocarbon than in the livers of the other animals used.

Preparation of unsaponifiable matter from 2 cwt. of pig's liver. Batches of ½ cwt. of pig's liver were treated in the manner described above for the preparation of the unsaponifiable matter. The yields obtained were not as quantitative as in the smaller preparations, owing to the difficulty of working up the material in bulk. The unsaponifiable matter obtained was stored in flasks completely filled with light petroleum. The livers used had come from animals which had been grass fed, and the unsaponifiable matter was an oily wax, dark red in colour. The four ½ cwt. batches of liver yielded 100, 94, 84, and 90 g. (mean 92 g.), which is 0·36 % of the wet weight of the liver.

Purification of the crude unsaponifiable matter. Squalene itself is miscible in all proportions with light petroleum, but its solubility in methyl alcohol is low (1·43 g. per 100 cc. at 15°). If the hydrocarbon present in these unsaponifiable matters bore any relation to squalene, therefore, its solubility in light petroleum and methyl alcohol should enable the bulk of the cholesterol to be removed, without appreciable loss of the hydrocarbon itself. This was investigated as follows: 1·3 g. of material from horse liver gave 0·113 g. of insoluble bromide; another 1·3 g. was boiled with 25 cc. methyl alcohol, cooled to room temperature and filtered; the filtrate after the usual treatment gave 0·041 g. of the bromide; the same quantity treated similarly with light petroleum instead of methyl alcohol gave 0·112 g. This experiment shows that extraction with light petroleum can be used for the removal of much of the cholesterol from the hydrocarbon without loss of the latter, and that the solubility of the substance in methyl alcohol at room temperature is very low.

The material, 368 g. in all, was therefore heated to boiling point with 1 litre of light petroleum and cooled. The mixture was shaken out on to a Buchner funnel and filtered; filtration was very slow and several changes of filter paper were necessary. The cholesterol remaining behind was washed

with light petroleum until it was free from the hydrocarbon. (Throughout this work we have used the formation of the insoluble bromide by the addition of bromine to an ether solution as indicating the presence of the hydrocarbon.) The cholesterol after drying *in vacuo* weighed 188 g. To ensure that the insoluble bromide formation was not due to the presence of traces of unsaturated fatty acids or to the presence of unhydrolysed fat from the original liver, the material contained in the light petroleum was resaponified with 25 g. of sodium dissolved in 350 cc. of alcohol in a current of nitrogen for 2 hours and for a further hour after the addition of 25 cc. of water. The usual ether-extraction was then carried out but considerable difficulty was encountered in washing the ether extract owing to the formation of emulsions. These emulsions have given trouble before when we have been dealing with ether solutions of squalene. The weight of material after this third saponification was 147 g. By bromination of 10 g. there was obtained 11·7 g. of bromide and, as the bromide contains 68 % bromine, the purity of the material giving rise to it must be 37·4 %, provided that one substance is giving rise to the bromide and that the yield is quantitative. The preparation of the hydrochloride from a known weight of material resulted in a yield corresponding to the original substance being 37·6 % pure. If we take the percentage purity therefore as 37·5, the 147 g. of the concentrated material must have contained 55 g. of the hydrocarbon; as this 55 g. originated from 2 cwt. of liver, the liver must have contained approximately 0·05 % of its wet weight as the hydrocarbon. It is to be noted that there is close agreement in the figures obtained for the purity of the material as given by the yields of hydrochloride and bromide. Further purification of this material, which was 37·5 % pure, was carried out by extraction with methyl alcohol. 5 g. were boiled with 150 cc. of methyl alcohol, cooled to room temperature and filtered. The insoluble material was a clear dark reddish-brown oil, and weighed 2·9 g. A determination of the weight of insoluble bromide obtained from a known weight of this 2·9 g., showed that it was now 63 % pure, for 0·29 g. yielded 0·57 g. of bromide. The substances extracted by methyl alcohol contained 27 % of cholesterol (digitonin method) and yielded only 0·3 g. of the bromide. The iodine value of the insoluble portion, 63 % pure, was 255 (Wijs); this fraction was again treated with 100 cc. of boiling methyl alcohol and the mixture cooled and filtered. The insoluble residue weighed 1·72 g., and 0·21 g. of it yielded 0·50 g. of the bromide corresponding to a purity of 76 %. Repetition of the methyl alcohol extraction, using 25 cc. of the solvent and filtering while the solution was hot, yielded 1·38 g. of the insoluble material and 0·07 g. of substance soluble in methyl alcohol. The former appeared to be 78 % pure by the method already adopted, and it was clear that purification by methyl alcohol extraction had been carried to its limit, for the last treatment had resulted in an increase of purity of only 2 %. This material therefore consisted of 78 % of the hydrocarbon and the remainder of other substances insoluble in methyl alcohol. It was a thick viscous oil, dark red in colour. Treatment of this

residue by boiling with charcoal in benzene solution resulted in a loss of material without any increase in purity.

The yield of squalene dodecabromide obtained by the addition of bromine to an ether solution of squalene varies from 22–45 % and the fact that the purity of this material as determined by the formation of the insoluble bromide was 78 % seems to be further evidence that the hydrocarbon is not squalene.

As methyl alcohol extraction had increased the purity of the substance from 37 to 78 %, it was decided to treat the main bulk in a similar manner. From the remaining 115 g. of thrice saponified material, a further 13 g. of cholesterol was removed by crystallisation from light petroleum. The remaining 102 g. was boiled with 250 cc. of methyl alcohol and filtered on cooling; the residue was again boiled with 250 cc. of methyl alcohol and filtered at 40°. The combined methyl alcohol mother liquors yielded 15 g. of crude cholesterol, and, after removal of this cholesterol, were again used for extracting the insoluble residue as before. After further treatment with 500 cc. and 250 cc. portions of fresh methyl alcohol, there remained behind 45 g. of material insoluble in methyl alcohol, whilst the methyl alcohol mother liquors contained 35 g. of material. The iodine value of the methyl alcohol-insoluble fraction was 309, and of the soluble portion 181. The former contained approximately 70 % of the hydrocarbon, and it was decided to submit it to distillation.

Distillation of the purified material. After a preliminary experiment on a small amount of material this was carried out on 27 g. at a pressure of 2 mm. There were obtained 1 cc. distilling from 200–225° and 1·5 cc. from 234–242°. These two fractions contained the cholesterol present in the material. The first fraction gave a very strong reaction with arsenious chloride, and was the usual vitamin A fraction [Drummond, Channon, Coward, 1925]. No further distillate was obtained until the temperature of the bath was about 350°. Distillation then recommenced and three fractions were obtained over the apparent temperature ranges of 264–290°, 292–296°, and 301–316°. Much decomposition, as evinced by white fumes, was apparent and the pressure rose to 8 mm. None of these fractions gave the usual insoluble bromide nor did the undistilled residue. Fractions 3, 4, and 5 appeared to be terpenes. Fraction 3 was a light mobile oil with a penetrating lemon-like smell; fraction 4 was more viscous and the last fraction still more so. This experiment shows that the hydrocarbon cannot be distilled at a pressure of 2 mm., but that if the temperature be raised sufficiently decomposition occurs and what appears to be a mixture of terpenes results. It was apparent that much of the distillate had been lost on account of lack of efficient condensation, for the material actually obtained was redistilled at 12 mm. with the following results:

	Temp. °C.	N_D^{20}	d_{20}^{20}
1.	65–100	1·4861	0·8672
2.	100–130	1·5020	0·8933
3.	130–200	1·5048	0·9020
4.	200–240	1·5092	0·9123
5.	240–270	1·5122	0·9222

Analysis of fractions 1, 2, and 4 gave the figures:

1. C, 85·2 %; H, 11·5 %; O (by difference), 3·3 %.
2. C, 86·2 %; H, 11·8 %; O „ 2·0 % (calculated for C_5H_8: C, 88·23 %; H, 11·77 %).
3. C, 84·7 %; H, 11·5 %; O „ 3·8 %.

These analyses show that the fractions contain oxygen, which is to be expected, for they were not analysed until some weeks after distillation and the readiness with which this type of compound takes up oxygen is well known. They would, however, seem to be of similar type to those obtained by Staudinger [1922] by the dry distillation of caoutchouc in a high vacuum and by Heilbron and Kamm [1926] by distillation of squalene at atmospheric pressure. It is of interest to note that squalene itself boils at 240° at 2 mm. and as the hydrocarbon with which we are dealing does not appear to distil at that pressure, it seems unlikely that it is squalene. The fact that the material which was used for the distillation was only 70 % pure prevents any rigid conclusion being drawn, but it would appear that the hydrocarbon may be closely related to squalene.

Analysis of the hydrochloride and bromide. The analytical figures for a number of preparations of these two derivatives are given in the following table. The hydrochloride was in each case recrystallised twice from acetone and washed with ether; it melted at 128°; the bromide was prepared as already described and ground with ether several times. Another preparation of the bromide (2 g.) was dissolved in 100 cc. of cold chloroform, and precipitated by the addition of 400 cc. of ether, and gave similar analytical figures. This bromide, like that of squalene, is insoluble in most solvents, but is somewhat soluble in pyridine, carbon disulphide, chloroform and tetrachloroethane; it does not melt, but darkens at 170° and goes black at 180°.

	Bromide			Hydrochloride		
	C	H	Br	C	H	Cl
	28·00 %	3·28 %	68·16 %	58·46 %	8·79 %	31·53 %
	28·33	4·08	67·76	58·81	8·81	30·83
	28·37	3·98	67·62	59·26	8·93	30·74
	28·50	3·99	67·90			
Mean	28·40	3·83	67·86	58·84	8·84	31·03

From these mean values it would appear that the bromide is pure; the figures for the hydrochloride show that that substance is only 98·71 % pure. We have been unable to obtain a purer product by repeated recrystallisation of the substance from hot acetone, for the crystals are contaminated by a brown oily material which washing with a large number of solvents has failed to remove. As to the nature of this impurity we have no information, but the hydrochloride, when first filtered after preparation, is black and although the bulk of this material can be removed by washing with ether and acetone, the small quantity remaining clings tenaciously to the hydrochloride and is not removed by recrystallisation. It would seem probable that this impurity is the result of partial decomposition of the hydrochloride itself. The microscopic appearance of the hydrochloride has already been mentioned. It is to

be pointed out, however, that the corresponding squalene derivative, which crystallises usually in hexagonal and diamond-shaped plates, does also give rise to needles very similar to those given by the hydrocarbon, but the former appears to be the more stable form. We have tried to obtain the hydrochloride of the new hydrocarbon in plate form similar to that of squalene hydrochloride and have failed repeatedly.

Unfortunately we have been able to determine the molecular weight neither of the hydrocarbon, on account of its impurity, nor of its derivatives, since neither the hydrochloride nor the bromide can be used cryoscopically on account of their low solubilities in any suitable solvents, whilst their tendency to decompose prevents use being made of the boiling point method or that based on the depression of the freezing point of camphor.

Some attempt has been made to regenerate the parent hydrocarbon from the hydrochloride and bromide. Squalene hydrochloride can be decomposed by boiling with sodium ethylate in alcoholic solution or by boiling with pyridine; it is even decomposed into its components by prolonged boiling in alcohol only [Heilbron and Kamm, 1926]. The hydrochloride has been submitted to the first two treatments with precautions taken to prevent oxidation, but, although the resultant oils have been highly unsaturated, they have failed to give solid products on the addition of bromine.

The chemical nature of the hydrocarbon.

It will be seen that very little evidence has been obtained as to the chemical nature of the hydrocarbon. The percentage composition of the hydrochloride and bromide are similar to those of the corresponding squalene derivatives, but with definitely lower halogen figures. In our opinion sufficient evidence has been adduced to enable us to say that the hydrocarbon is not squalene itself, and a number of facts suggest that it is possibly a higher homologue of that substance. Thus the hydrochloride is much less crystalline and its melting point is higher; the hydrocarbon does not distil at a pressure of 2 mm. but breaks down into a mixture of terpenes, whilst squalene itself distils at 240°/2 mm., but if distilled at atmospheric pressure, it also breaks down and seems to give rise to what appear to be very similar products [Heilbron and Kamm, 1926]. It is obvious that this evidence from the behaviour on distillation must not be regarded as being too definite, for the material distilled was only 70 % pure and the small amount of the products of distillation has prevented their study in any detail. There are given in the following table the percentage compositions required by derivatives of squalene and possible higher homologues and average figures obtained on those of the hydrocarbon.

	C	H	Br		C	H	Cl
$C_{30}H_{50}Br_{12}$	26·29 %	3·68 %	70·03 %	$C_{30}H_{56}Cl_6$	57·18 %	8·96 %	33·86 %
$C_{32}H_{54}Br_{12}$	27·47	3·86	68·67	$C_{32}H_{60}Cl_6$	58·42	9·20	32·34
$C_{33}H_{56}Br_{12}$	28·05	4·00	67·94	$C_{33}H_{62}Cl_6$	59·02	9·31	31·68
$C_{34}H_{58}Br_{12}$	28·62	4·10	67·28	$C_{34}H_{64}Cl_6$	59·55	9·40	31·02
X Br	28·40	3·83	67·86	*X* Cl	58·84	8·84	31·03

It will be seen from these figures that the addition of one carbon atom to the molecule does not entail a marked difference in the percentage figures, and as the amounts of the derivatives with which we have been dealing have been rather small, it would be wise not to lay too much stress on the results given by them, but to leave the question open until surer methods of purification have been devised. This preliminary note has been published because the preparation of a large amount of material will take some considerable time, and we are hoping to find a source of the hydrocarbon which will enable us to obtain it less contaminated with pigments and other substances having like solubilities. Hydrogenation may then result in the production of the corresponding saturated substance which may be distillable, since hydrogenated squalene, $C_{30}H_{62}$, distils at a lower temperature than squalene itself.

Note. Very large volumes of solvent have necessarily been used in the preparation of the material. Thus for each batch of ½ cwt. of liver some 20 l. of ether and 4 l. of absolute alcohol have been used. It was considered wise therefore to carry out a test on the reagents. Accordingly 10 l. of 5 % KOH solution were extracted with 10 l. of ether and 2 l. of alcohol and the extract treated in the same way as when dealing with the liver pulp. After drying at 100° *in vacuo*, there remained behind 0·11 g. of oil which did not give a solid bromine derivative. It was considered unnecessary to submit this to a second saponification. It has been our practice to use absolute alcohol, which has been treated with sodium and refluxed for many hours and then redistilled for the second saponification in order to minimise the amount of resinous material contaminating the final product. The residue from 5 l. of this alcohol to which had been added 200 g. of sodium over the course of several weeks and which was deep red in colour, was thrown into water and ether extracted in the usual way. It yielded 0·10 g. of a dark resin which, though unsaturated, did not give a solid bromine derivative. Another similar test carried out with a view to forming the hydrochloride also gave a negative result, as did a mixture of synthetic glycerides and cholesterol derivatives which was put through the complete process.

Summary.

1. There is present in the liver of man, ox, sheep, horse, and pig a highly unsaturated hydrocarbon which has not been isolated in a pure state.
2. The hydrocarbon yields a crystalline hydrochloride and a solid addition product with bromine. Analyses of these derivatives are given.
3. The hydrocarbon does not distil at a pressure of 2 mm., but decomposes, yielding what appear to be terpenes.
4. From a consideration of the results obtained it appears probable that the hydrocarbon is not squalene ($C_{30}H_{50}$), but a substance closely allied to it.
5. Squalene is apparently absent from the livers investigated.

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