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

III. FISH-LIVER OILS.

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In a previous paper [Channon, 1926] it was reported that when the unsaturated hydrocarbon squalene was administered to rats, partial absorption of the hydrocarbon occurred. At the same time, there was a considerable increase in the amount of the unsaponifiable fraction of the liver, and the presence of squalene could be demonstrated in that fraction. The increase in the amount of the unsaponifiable matter of the liver was chiefly caused by a rise in the amount of cholesterol to more than twice its normal value. This marked rise in liver cholesterol suggested that squalene or some substance of similar chemical nature might be the precursor of cholesterol in the animal body. On the other hand, the increase in the amount of the unsaponifiable fraction was not entirely accounted for by the amount of squalene present and the increase in cholesterol. It thus appeared possible that the increase in sterol was the result of some process existing for the purpose of keeping the ratio of the amounts of cholesterol to that of the other unsaponifiable substances present as constant as possible. It was with a view to obtaining further information on these points that the work described in this paper was undertaken.

It seemed probable that a study of the amount of sterol present in the unsaponifiable fractions of a number of liver oils might throw some light on the question of a possible relationship existing between the amounts of sterol and those of the other unsaponifiable substances present. Fish-liver oils were chosen for this purpose for a number of reasons. Firstly, there is present in a number of these oils a high percentage of unsaponifiable matter and the study of these should make any such relationship the more apparent. Secondly, it was desired at the same time to obtain information regarding the distribution of squalene, for although the amount of the hydrocarbon present in a large number of sharks of Japanese waters has been investigated by Tsujimoto and Toyama [Tsujimoto, 1916, 1918, 1920, 1; Toyama, 1922, 1923, 1] no observations as to its presence in the commoner fish of British waters are available. Further, evidence as to the possibility of squalene being the precursor of cholesterol in the animal body might be forthcoming as the result of a study of the amount of sterol in the squalene-containing oils.

Another question on which information has been sought is the source from which squalene is derived. Whether certain fish are able to synthesise squalene or whether the hydrocarbon is derived from the food is a question which cannot be readily answered. A similar type of experiment to that reported on cholesterol synthesis in the rat [Channon, 1925] suggests itself, but unfortunately the fact that the squalene-containing fish available in home waters are viviparous renders such an experiment almost impracticable. It was desirable, therefore, in addition to determining whether the hydrocarbon is present in fish-liver oils generally, to prepare the unsaponifiable fraction of a number of samples of plankton in order to determine whether this material, which is the ultimate food of fish, contained it.

With these problems in view, the unsaponifiable matter has been prepared from a number of fish-livers and fish-liver oils: the sterol contents of these materials have been determined, and they have been examined for the presence of squalene: similar observations have been made on a number of samples of plankton and a comparison has been drawn with the unsaponifiable fraction of certain mammalian livers.

Experimental.

The livers of the fish used in this work were obtained by the author from the Marine Biological Station, Plymouth. The livers of the fish caught in shallower waters were removed immediately from the fish as soon as landed by the trawler belonging to the Station. Those of the fish caught in deeper waters were procured in the fish market from the fish freshly landed from the deep sea trawlers. As the latter are at sea for varying periods, the livers of these fish were seldom fresh.

In attempting to discover whether squalene was present in the livers used, it was clearly desirable first to prepare the unsaponifiable fraction of these oils because, since the proportion of unsaponifiable matter in the majority of them is very low, the attempt to find squalene in the original oil might fail on account of the small quantities present. Accordingly, the livers of the fish after weighing were covered with 5 % aqueous potassium hydroxide and stored in well-stoppered bottles until opportunity arose for continuing the extraction, which in most cases was several weeks after the material had been brought to London.

Preparation of the unsaponifiable matter.

The methods used for the preparation of the unsaponifiable matter were those already described by the author [Channon, 1925]. Briefly, after complete solution of the livers in the hot aqueous 5 % potassium hydroxide and considerable dilution of the resulting fluid with water, an exhaustive ether extraction was made. After washing with water, the ether extract was evaporated to dryness, and the resulting oil dissolved in hot alcohol and re-saponified with sodium ethoxide. The fluid obtained by pouring the solution of the re-saponified product into water was again exhaustively extracted with ether,

the ether extract was washed with water and evaporated to dryness *in vacuo*. In this way there was obtained the unsaponifiable fraction. In a number of cases where large amounts of liver were used, it was necessary to carry out a third saponification on account of the high content of oil of some of the livers. It is clear that the extraction of an aqueous digest of liver tissue in bulk cannot be strictly quantitative. There are troubles due to emulsions which are not easily overcome. Hence, although up to twenty ether extractions were made in some cases, it is not claimed that the yields obtained were always the maximum possible. There is probably in these cases a loss of not more than 5 %. This is not considered of importance here, as the work was designed to obtain figures showing what livers, if any, contained a large amount of unsaponifiable matter, and losses of this order may be neglected. It may be taken also that the losses were general and not losses of the less soluble constituents, so that the percentages of sterol present may be regarded as sufficiently accurate for the purposes planned.

A number of the oils used were kindly supplied by Prof. I. M. Heilbron of Liverpool University. These were saponified twice, first with alcoholic potassium hydroxide and then with sodium ethoxide. The amount of sterol present in the unsaponifiable fractions was determined on aliquot portions by the digitonin method of Windaus [1910]. The possible presence of squalene was sought by attempting to prepare the characteristic crystalline hexahydrochloride $C_{30}H_{56}Cl_6$ in the following manner. The solution of unsaponifiable matter was evaporated to dryness *in vacuo* and then dissolved in the minimum amount of boiling water-free acetone. The solution was allowed to cool and then filtered rapidly. In this way, a large proportion of the sterol present was removed and the other substances present were obtained in strong solution in acetone. These cooled acetone solutions were then saturated with dry hydrochloric acid gas. After cooling on ice, any precipitate was filtered off, washed with ether and recrystallised from acetone. Its characteristic crystalline form and melting point were used for the identification of the hydrochloride. Acetone was used as a solvent for the preparation of the hydrochloride in preference to the solvents previously used [Channon, 1926; Drummond, Channon and Coward, 1925] on account of the higher yields so obtained [Heilbron, Kamm and Owens, 1926]. Exactly similar methods were used for the preparation and subsequent treatment of the unsaponifiable fraction from the plankton. In those cases where the percentage of the unsaponifiable fraction was high its iodine value was determined.

Results.

The weights of liver or liver oil used and the yield of the unsaponifiable fractions, together with the sterol calculated as a percentage of the unsaponifiable fraction, are given in Table I.

By the kindness of the Director of the Marine Biological Station, Plymouth, a number of samples of plankton were placed at the author's disposal. The

Table I.

Order SELACHII Sub-order	Family			Liver g.	Liver oil g.	Unsap. matter g.	Unsap matter as % of liver or oil	Sterol as % of unsap. matter	Iodine value
Notidani	Notidanidae	*Notidanus griseus*	six-gilled shark	—	8·03	1·1184	13·92	44·8	72·4
Scylloidei	Scylliidae	*Scyllium canicula*	spotted dogfish	410	—	1·3432	0·328	64·0	—
				—	6·63	0·1270	1·915	44·7	—
	Lamnidae	*Lamna cornubica*	porbeagle	—	10·22	0·1150	1·126	40·9	—
	Charchariidae	*Mustelus vulgaris*	smooth hound	200	—	1·0010	0·500	39·3	—
		Galeus vulgaris	tope	—	7·76	0·2968	3·824	20·46	—
Squaliformes	Squalidae	*Spinax niger*	spinax	—	8·55	5·7387	67·13	0·61	343·0
		Acanthias vulgaris	picked dogfish	—	7·75	0·9675	12·48	7·96	67·4
		Scymnodon ringens		—	8·35	2·7969	33·50	1·47	73·7
		Scymnorhinus lichia	darkie Charlie	—	8·90	7·2541	81·49	0·13	341·5
		Lepidorhinus squamosus		—	8·72	6·3539	72·88	0·53	362·7
Raiiformes	Squatinidae	*Rhina squatina*		815	—	4·6420	0·57	72·6	—
	Torpedinidae	*Torpedo nobiliana*	electric ray	—	8·94	1·4867	16·63	10·59	79·5
	Raiidae	*Raia batis*	skate	—	10·48	0·2210	2·11	51·45	—
		,, *circularis*	sandy ray	500	—	0·9288	0·185	64·5	—
		,, *clavata*	thornback ray	2500	—	8·535	0·341	38·2	—
				—	9·22	0·2287	2·48	24·66	—
		,, *sp.*	black skate	1130	—	9·060	0·802	64·7	—
Order TELEOSTEI									
Anacanthini	Gadidae	*Gadus luscus*	bib	45	—	0·1568	0·348	46·4	—
		,, *merlangus*	whiting	27	—	0·1198	0·444	42·9	—
		,, *minutus*	poor cod	185	—	0·8192	0·443	81·4	—
		,, *pollachius*	pollack	80	—	0·3560	0·445	65·9	—
		,, *virens*	coalfish	930	—	2·0793	0·224	59·4	—
		Merluccius vulgaris	hake	—	10·34	0·1443	1·397	56·4	—
		Phycis blennoides	greater forkbeard	—	7·20	0·1702	2·363	51·4	—
Apodes	Anguillidae	*Conger vulgaris*	conger	745	—	2·5868	0·347	58·2	—
Acanthopterygii	Triglidae	*Trigla cuculus*	red gurnard	165	—	1·2082	0·731	83·5	—
		,, *gurnardus*	grey gurnard	65	—	0·7358	1·132	63·9	—
		,, *lineata*	streaked gurnard	—	—	0·0507	—	73·2	—
	Pleuronectidae	*Pleuronectes platessa*	plaice	91	—	0·4138	0·455	74·9	—
		,, *microcephalus*	lemon sole	65	—	0·0664	0·102	54·2	—
	Callionymidae	*Callionymus lyra*	dragonet	80	—	1·3462	1·683	69·3	—
Pediculati		*Lophius piscatorius*	angler	420	—	2·6686	0·636	68·9	—

unsaponifiable fractions from these and also from a further sample obtained from the Scottish Marine Biological Station, Keppel Pier, Millport, Bute, were prepared. The percentage of sterol precipitated by digitonin and the iodine values of the sterol-free fractions are given in Table II.

Table II.

Source and nature of plankton		Wt. of unsap. fraction g.	Sterol as % of unsap. fraction	Iodine value of sterol-free material
Millport.	Large animal	4·57	0·45	50·5
Plymouth.	Large animal	0·1384	29·48	106·3
	Medium animal	0·0804	12·63	77·8
	Small animal and some plants	0·5436	21·20	75·1
	Mostly plant life: diatoms	0·1206	7·49	90·2

Squalene was not found in any of these fractions. One point of interest regarding the unsaponifiable fraction of the specimen obtained from Millport is that, although carefully prepared in a current of nitrogen, it failed to give the vitamin A reaction with arsenious chloride [Rosenheim and Drummond, 1925].

Discussion.

1. *The distribution of squalene.*

Squalene was not found in any of the unsaponifiable fractions examined save in those from the livers of three members of the Squalidae family—*Spinax niger*, *Scymnorhinus lichia* and *Lepidorhinus squamosus*, in which the percentages of unsaponifiable matter were 67·13, 81·49 and 72·88 respectively. This limited distribution of the hydrocarbon makes it of interest to compare the results of Tsujimoto [1920, 1], who found it present in the livers of 16 of the 36 species of the Elasmobranchs of Japanese waters which he examined. Only in liver oils having a specific gravity of less than 0·9 at 15° was squalene present in quantity; in three oils of a slightly greater specific gravity, it was present in small amounts only. It is present chiefly in the liver oils of the family Squalidae, but is also present in certain members of the Cetorhinidae, Chlamydoselachidae, Dalatiidae and Scylliorhinidae. The percentage of unsaponifiable matter in the oils of specific gravity of less than 0·9 may vary from 37 to 90·2 %, with iodine values from 130 to 345; in those of higher specific gravity, the percentage of unsaponifiable matter varies from 0·9 to 23·2 % and the iodine values from 91 to 197. From these results it is seen that squalene is found chiefly in the Squalidae, although curiously enough not every member of that family contains it. This curious distribution of the hydrocarbon is perplexing. It is unlikely that squalene is derived from the food for, if such were the case, it seems probable that it would be present in the livers of fish generally, and yet not only is it limited in its distribution, but it is absent from those samples of plankton examined. Further, the fact

that the feeding habits of all the members of the Squalidae family appear similar is also evidence against it being a product derived from the food. Nor does it seem reasonable to suppose that a fish should possess the power of replacing the greater part of its liver oil by a hydrocarbon derived from the food. On the other hand, if the hydrocarbon is synthesised by certain fish, it is also difficult to understand its distribution or to account for its presence in very large amounts in the livers of certain of the Squalidae and its absence from others.

Another point worthy of notice is that squalene also occurs in the egg oils of *Chlamydoselachus anguineus* and *Lepidorhinus kinbei* [Tsujimoto, 1920, 2]. Further, Heilbron, Kamm and Owens [1926] reported that they examined 115 female *Etmopterus spinax*. In some of these eggs in a comparatively undeveloped condition were found and all contained squalene. In others, where development had proceeded far, the yolk sac was devoid of squalene, showing that absorption of the substance had occurred during development.

The absence of squalene from the Teleostei is interesting, for it will be remembered that it was reported to be present in very small amounts in cod-liver oil [Drummond, Channon and Coward, 1925], and Weidemann [1926] confirms the presence of the hydrocarbon in that oil. It is possible, however, that the hydrocarbon is not present in cod-liver oil, other than fortuitously, for in the degutting of the cod at sea, it is usual for the livers of fish other than cod to be mixed with the cod-livers themselves.

2. *The yield of unsaponifiable matter.*

The figures presented show that there is a large variation in the amount of unsaponifiable matter yielded by the livers of different fish. Strict comparison between the Selachii and Teleostei is not possible, as in the former the liver oils were used in most cases and in the latter the livers themselves. Certain facts stand out, however. It is only among the Selachii that there are found fish with a high content of unsaponifiable matter in their livers. Thus, among the Squalidae, in the three fish in which squalene is present, the values for the percentages of unsaponifiable matter are 67·13, 81·49 and 72·88 %. Such high values, however, are not confined to the squalene-containing fish, for there are present in the liver oils of *Notidanus griseus* 13·92, *Acanthias vulgaris* 12·48, *Scymnodon ringens* 33·5 and of *Torpedo nobiliana* 16·63 % of unsaponifiable matter. As to the nature of the substances other than squalene present in these liver oils, reference may be made to the work of Tsujimoto and Toyama [1922] who found 15·42 % of unsaponifiable matter in the liver oil of *Hexanchus corinus*. Squalene was not present, the chief constituents being batyl and selachyl alcohols and these alcohols were present in the liver oils of *Cirrhigaleus barbifer, Somniosus microcephalus, Narcacion takionis, Chimaera owstwonii, C. mitsukurii, Lepidorhinus kinbei and Zameus squamulosus*. Toyama [1924, 1] found these same alcohols present in *Chimaera barbouri* and also reported the presence of chimyl alcohol $C_{19}H_{40}O_3$; they are also

present in the liver oils of a number of other fish [Toyama 1923, 1; 1924, 2]. Further, the presence of oleyl and cetyl alcohols has also been reported, for Toyama [1923, 1] found the unsaponifiable matter of the liver oil of *Chlamydoselachus anguineus* to consist chiefly of oleyl alcohol together with small amounts of cetyl alcohol and cholesterol.

In addition to squalene and the alcohols already discussed, there may be present a saturated hydrocarbon, for Tsujimoto [1917] isolated *iso*-octadecane from the liver oil of *Cetorhinus maximus*, and Toyama [1923, 2] found this hydrocarbon in a number of liver oils.

Another point worthy of notice is that the amount of unsaponifiable matter in any given liver oil may vary considerably. Toyama [1922] reported that the liver oil of *Chlamydoselachus anguineus* contained percentages of unsaponifiable matter varying from 37·06 to 51·65 %; the oil from *Scymnorhinus lichia* yielded 48·51 % while the writer found 81·49 %.

Sufficient has been said to illustrate the interesting problems which are raised by the presence of alcohols and hydrocarbons in such quantities in these fish-liver oils. Evidence concerning the function of these substances and of the chemical processes involved in their utilisation is urgently required, for such evidence may throw fresh light on our conceptions of fat metabolism.

It is of interest at this point to compare the yields of unsaponifiable matter obtained from various mammalian livers expressed as g. % of the liver. Cat 0·32, 0·56, 0·87, 0·38, 0·29; ox 0·35; pig 0·32; horse 0·25; human 0·35, 0·67, 0·58, 0·98; figures for rats' livers have been recorded [Channon, 1926]. These materials were in all cases solids containing much cholesterol, which usually amounted to about 70 % of the total.

3. *The proportion of sterol in the unsaponifiable fractions.*

Just as there are wide differences between the Selachii and Teleostei in the amount of unsaponifiable matter in the livers, so are there striking variations in the percentages of sterol present in these fractions. Among the Teleostei, the extreme values for the amount of sterol expressed as a percentage of the unsaponifiable fractions are 42·9 % and 83·5 % with a mean value of 63·3 %. These may appear wide variations but the figures obtained are of the same order, and, since the sterol constitutes a considerable proportion in all these particular unsaponifiable fractions, this mean value of 63·3 % has some significance.

If we consider the oils from the livers of the Selachii, however, a number of curious values obtain for the sterol content, *i.e.* from 0·13 % to 51·45 % with a series of intermediate values. These values appear at first sight to bear no relation to one another, but if we arrange the fish in order so that those with the greatest content of unsaponifiable matter in the liver appear at the top, and also record the percentage of sterol in this unsaponifiable fraction, we obtain the following result (Table III).

Table III.

Fish	Unsaponifiable matter %	Sterol as % of unsaponifiable matter
Scymnorhinus lichia	81·49	0·13
Lepidorhinus squamosus	72·88	0·53
Spinax niger	67·13	0·61
Scymnodon ringens	33·5	1·47
Torpedo nobiliana	16·63	10·59
Notidanus griseus	13·92	44·78
Acanthias vulgaris	12·48	7·96
Galeus vulgaris	3·82	20·46
Raia clavata	2·48	24·66
„ *batis*	2·11	51·45
Scyllium canicula	1·91	44·70
Lamna cornubica	1·13	40·9

A curious result is obvious, namely that, in general, as the amount of unsaponifiable fraction of the liver oil increases, so does its sterol content decrease. The only obvious exception in the above table is in the case of *Notidanus griseus*, but as the unsaponifiable fractions were prepared from one sample of oil only in each case, this particular result may be fortuitous. The conclusion from the above table may be expressed in the general form that oils of approximately the same content of unsaponifiable material will contain approximately the same amount of sterol. It is obvious that such a relationship will not be detectable in the unsaponifiable fraction of those livers which contain only 1–2 % of unsaponifiable material. It is thus only among those given, *i.e.* the Selachii, where the unsaponifiable fraction is very high, that the relation becomes apparent, but presumably it is a general one. Such a relationship, in which the amount of sterol present in an oil varies inversely with the amount of the total unsaponifiable fraction of the oil, is one not easy to interpret. It seems less probable to the author that the relationship is one between the sterol and the total unsaponifiable fraction than that it is one between the sterol and the true fat present in the oil.

Further experiments are clearly necessary in which attention must be paid not only to the amount of unsaponifiable matter and sterol in the liver oil, but also the amount of oil yielded by the livers of different fish. The determination of the amount of oil that a given weight of liver contains becomes of importance when it is remembered that the livers of various fish contain markedly different percentages of oil, for although the figures recorded in this paper give the percentages of unsaponifiable matter and sterol in a given liver oil, they do not give any idea as to the total amount of sterol and fat present in the liver itself.

It was stated in the introduction to this paper that one reason for studying the sterol percentages was to obtain light on a possible relationship between squalene and sterol. The evidence given in the above table is quite definite in showing that the very low percentage of sterol in the squalene-containing livers is not due to the presence of squalene as such but to the quantity in

which it is present, for the relationship between sterol and the unsaponifiable fraction is maintained throughout whether squalene is present or not. Thus the low percentage of sterol in the squalene-containing oils is merely due to the mass relations existing between the sterol and the rest of the unsaponifiable fractions, and hence this low percentage of sterol is no evidence whatever of any relation between sterol and squalene.

SUMMARY.

1. A study has been made of the yields of unsaponifiable matter from the livers of a number of fish.

2. The liver oils of the Selachii differ from those of the Teleostei in that, in many cases, very large amounts of unsaponifiable matter occur in the former.

3. A relationship seems to exist between the percentage of unsaponifiable matter in the liver oils of the Selachii and their sterol content. The higher the percentage of unsaponifiable matter in a given oil, the lower is the percentage of sterol in that fraction.

4. Squalene was not detected in the liver oils of any of the fish studied, save in those from three members of the Squalidae family.

5. The question as to whether squalene is synthesised or is derived by the fish from its food is briefly discussed.

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