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

I. EXPERIMENTS WITH THE UNSATURATED HYDROCARBON, SQUALENE (SPINACENE).

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THIS paper is concerned with some feeding experiments on rats with squalene, a hydrocarbon of the formula $C_{30}H_{50}$ occurring in certain shark-liver oils, described by Tsujimoto [1916]. In a later paper [1920] he describes investigations carried out on 36 species of sharks, and it was found that the hydrocarbon occurred in the liver oils of numerous species and that these liver oils had in general a specific gravity of less than 0.9. Only in a very limited number of cases where the specific gravity of the oils was greater than 0.9 was squalene found and then in small quantity only.

Meanwhile Chapman [1917] independently isolated a hydrocarbon from the liver oils of *Centrophorus granulosus* and *Scymnorhinus lichia*; he gave it the name spinacene and attributed to it the formula $C_{30}H_{50}$. Later, however [1918, 1923], from a consideration of further results which had been obtained with larger quantities of material, he expressed the view that the formula of the substance should be $C_{29}H_{48}$ and not $C_{30}H_{50}$. Referring to Chapman's belief that the formula of spinacene was $C_{29}H_{48}$, Tsujimoto [1920] says: "Considering the fact that I have already prepared the hexahydrochloride of squalene of the formula $C_{30}H_{50}6HCl$, which has properties very similar to spinacene hexahydrochloride, $C_{29}H_{48}6HCl$, and also that spinacene has been obtained from an oil of the *Squalidae* sharks, it is highly probable that spinacene is identical with squalene." Further, in studies on the chemical nature of vitamin A, Drummond, Channon and Coward [1925] obtained very small quantities of the hexahydrochloride of the hydrocarbon from the unsaponifiable matter of cod-liver oil. Preparations of the hexahydrochloride were made from pure spinacene, and the highest value obtained for chlorine was 33.61 %, which agrees much better with $C_{30}H_{50}6HCl$ (Cl 33.86 %) than with $C_{29}H_{48}6HCl$ (Cl 34.63 %). These authors therefore also incline to the view that spinacene and squalene are identical and that the formula of the substance is $C_{30}H_{50}$. Other evidence points in the same direction; Prof. I. M. Heilbron, of the University of Liverpool, who has been working on this hydrocarbon and whose

results are about to be published [Heilbron and Kamm, 1926], has kindly consented to our making mention of such of his results as have a bearing on this work.

From a long and careful study of the hydrochloride of the hydrocarbon obtained from the liver oil of *Scymnorhinus lichia*, from which Tsujimoto isolated "squalene" ($C_{30}H_{50}$) and Chapman "spinacene" ($C_{28}H_{48}$), Heilbron and Kamm also arrive at the conclusion that squalene and spinacene are one and the same substance of the formula $C_{30}H_{50}$. As the bulk of the evidence points to the identity of these substances, and as "squalene" was described first [Tsujimoto, 1916], it is proposed to refer to the hydrocarbon in this paper as squalene and to adopt the formula $C_{30}H_{50}$.

Squalene is a colourless liquid hydrocarbon containing six double bonds. It forms a crystalline hexahydrochloride which is its most characteristic derivative. The melting point of this compound varies according to the method adopted in its recrystallisation (from acetone), but the usual preparation results in a substance melting at 113° . The crystals are hexagonal and diamond-shaped plates, but on rapidly cooling a solution in acetone fine needles may be obtained which appear to change into the plate form. The reasons for the variability of the melting point of the hydrochloride are given by Heilbron and Owens [1926]. This hydrochloride serves for the detection of the parent substance and reference has already been made [Drummond, Channon and Coward, 1925] to the fact that it can be obtained in variable yield by passing dry hydrochloric acid gas into a solution of squalene in a suitable solvent. Hitherto we have used ether and alcohol but a better yield can be obtained if acetone be used. Both these methods have been employed in the course of this work.

The occurrence of an unsaturated hydrocarbon in the liver oils of various fish is of peculiar biological interest. Tsujimoto [1920] gives a list of the 36 species of shark which he has examined; squalene is found chiefly in the *Squalidae*, but not every member of the order contains the hydrocarbon, which is also widely distributed among other Elasmobranchs. The amount of the hydrocarbon present in the liver oil varies very greatly. Usually fish-liver oils contain 1 to 2 % of unsaponifiable matter only, but the liver oils of some of the fish referred to may contain as much as 80 %, nearly all of which is squalene. It will be clear that this striking difference in the composition of the liver oil, which may contain only 20 % of true fat, must necessarily entail some abnormal metabolism which has not been hitherto described. Possibly squalene is formed as an intermediate compound which normally undergoes degradation into other products, and it may be that this process is for some reason or other hindered, with the resulting accumulation of the hydrocarbon. One important fact to be established is whether or not squalene is synthesised by the organism or is derived from the food ingested, and to this question we are turning attention. Further, the hydrocarbon appears not only in the livers of the fish, for Tsujimoto [1920] found it present in the egg oil of

Chlamydoselachus anguineus and *Lepidorhinus kinbei*. Heilbron and Kamm have also found squalene present in the eggs of *Etmopterus spinax*, *Lepidorhinus squamosus* and *Scymnorhinus lichia*. They examined 115 female specimens of *Etmopterus spinax* and in a number of them comparatively undeveloped eggs were found to contain squalene; in others in which development was almost complete, the yolk-sacs were found devoid of squalene, so that the substance was presumably absorbed during the course of development.

Reference is made in the next paper to the fact that there is a highly unsaturated hydrocarbon also present in the livers of man, ox, sheep, horse and pig. From results which have been so far obtained it would appear that this hydrocarbon is probably not squalene but a substance closely allied to it. The finding of a hydrocarbon of similar chemical nature in the mammal would suggest that this type of substance may be generally distributed, may possibly be of definite physiological importance in the metabolism of fats or allied substances, and that the occurrence of squalene in such quantities in fish livers may be a less remarkable phenomenon than would appear at first sight.

The work described in this paper was carried out with a view to determining whether squalene was absorbed by the rat; its occurrence in fish-liver oils suggested probable absorption, but the fact of its being a hydrocarbon of high molecular weight was against this view.

EXPERIMENTAL.

Experiment 1.

The general plan of the experiment was to feed two groups of rats on a complete artificial diet, while one group received in addition a small quantity of squalene each day. The diet used was that described by Drummond and Coward [1920], and was given to animals which had been so chosen that each group contained an animal of the same sex and weight. Immediately before feeding-time the animals were given approximately 0.05 cc. of squalene dropped directly into their mouths from a micro-burette. Records were taken of the amount of squalene given each day. The faeces were collected throughout the experiment at weekly intervals and stored in alcohol. Some little difficulty was encountered at first in finding the requisite amount of squalene to give to each rat per day, for beyond a certain daily dosage the substance exerts a marked purgative action which is in contrast with the behaviour of liquid paraffin which acts merely as a lubricant. The amount given was never in excess of 0.075 cc. per rat per day.

Treatment of livers. At the end of the experiment the animals were killed by chloroform and their livers removed as free from blood as possible. The combined livers of each group were weighed, heated with 5 % potassium hydroxide solution until solution was effected and extracted six or seven

times with ether in a separating funnel after adding sufficient alcohol to ensure rapid separation of the two layers. The combined ether extracts of each batch of livers were washed with water to remove soap and alcohol, evaporated to dryness and finally dried *in vacuo* at 100°. Excess of sodium ethylate in alcohol was then added and a second saponification carried out. Usually the residue from the ether extract after the first saponification consisted chiefly of unsaponifiable matter. After saponification with sodium ethylate, the solution was thrown into water and extracted with ether as before. Great care was taken at this stage to ensure by many washings with water that no soap was left behind in the ether extract. The ether extract was dried *in vacuo* at 100° and the weight of the unsaponifiable matter obtained. Cholesterol was quantitatively removed by the method of Windaus [1910], by adding an excess of a 1 % solution of digitonin in 90 % alcohol to a solution of the unsaponifiable matter in 95 % alcohol. After standing overnight the digitonide was filtered off, washed with ether and water to remove the excess of unsaponifiable matter and digitonin respectively, and weighed in a Gooch crucible. The mother liquor containing the unsaponifiable matter other than cholesterol was thrown into water, and exhaustively extracted with ether as before. After the usual washing of the ether extract, the ether was removed and the residue weighed after drying at 100° *in vacuo*; it was then dissolved in ether dried over sodium, and the solution saturated with hydrochloric acid gas. After standing overnight on ice the solution was filtered and the residue, if any, washed with ether to remove the adherent dark oily materials. The material so obtained was then recrystallised from hot acetone, filtered off and weighed. It was examined microscopically and its melting point determined.

The bodies of the animals were treated similarly as regards saponification. Cholesterol was, however, estimated in a small aliquot part. Squalene was not looked for in the unsaponifiable matter from the bodies.

Treatment of faeces. The faeces, which had been stored in alcohol, were ground up in the alcohol and filtered; two more grindings with absolute alcohol were made and the residue sucked as dry as possible. The residue was then extracted with ether for 3 days, re-ground and re-extracted for 1 day. All the alcohol washings and ether extracts were combined, and the solvents removed. Two saponifications followed by ether extractions were carried out as already described. Determinations of sterol were made by the modification of the method of Windaus [1910] introduced by Fraser and Gardner [1910]. Iodine values were also determined (Wijs). In this experiment the animals were killed 2 hours after receiving their last daily amount of squalene. The experiment lasted 42 days and each animal received 2.77 g. of squalene.

It will be more convenient to consider the results of all the experiments together later. It is to be pointed out, however, that as the experimental animals had received their daily dose of squalene just prior to being killed and as their alimentary tracts were not removed prior to the digestion of their bodies with potassium hydroxide, this amount of squalene (0.768 g.)

will appear in the weight of the unsaponifiable matter of their bodies. This figure will also include some part at least of the squalene given on the previous day. Hence it would appear desirable to subtract from the figure obtained (4.4474 g.), 1.536 g., as representing the amount of squalene administered on the day of killing and the preceding one. This gives a figure 2.9114 g. as the weight of the unsaponifiable matter of the bodies of the group of animals receiving squalene.

Exp. 1. Duration of experiment, 42 days.

		Control diet		Control diet + squalene			
Number of animals		9		10.0			
Squalene given, g.		0		27.7			
Weight of livers, g.		64		78.0			
Weight of bodies less livers, g.		1230		1405.0			
		Livers		Bodies		Faeces	
		Control g.	Control + squalene g.	Control g.	Control + squalene g.	Control g.	Control + squalene g.
(a)	Weight of unsaponifiable matter	0.3680	1.0774	1.8620	4.4474	2.6152	20.6890
	Weight of cholesterol in (a)	0.2576	0.6189	1.3000	2.1620	1.2412	1.7150
(b)	Weight of material not cholesterol in (a)	0.1004	0.4585	0.5620	2.2854	1.3742	18.9740
	Weight of C ₃₀ H ₅₀ 6HCl obtained from (b)	0.00	0.14	—	—	—	—
	Iodine value of (a) (Wijs)	—	—	135.4	177.1	80.4	316

It is clear from the weight of unsaponifiable matter from the faeces of the latter animals, and from its iodine value, that much of the squalene administered has been excreted. If we assume that that fraction of this unsaponifiable matter which is not squalene has an iodine value which is the same as that of the material from the control group, namely 80.4, then we may calculate from the iodine value of the whole of this unsaponifiable matter (316), that the amount of squalene present is 81.1 %, *i.e.* 16.79 g. (squalene has iodine value 371). This figure can only be approximate, but some rough check on it is obtained from the fact that, if we subtract the weight of material not sterol in the faeces of the control group from the corresponding figure for the faeces of the animals receiving squalene, we arrive at the figure 17.60 g. These figures 16.79 and 17.60 g. can only be approximate, but they would seem to show that about 17 g. of the 27.7 g. of squalene administered had been excreted. Thus about 10 g. have been absorbed during the course of 42 days and this absorption has resulted in a marked increase in the weight of unsaponifiable matter in the livers and also in the bodies of these animals, namely, from 0.3680 to 1.0774 g. in the case of the livers and from 1.8620 to 2.9114 g. in that of the bodies. There is also a large increase in the amounts of cholesterol, from 0.2576 to 0.6189 g. in the livers and from 1.3000 to 2.1620 g. in the bodies. The results are considered in more detail later.

Experiment 2.

The work described in Exp. 1 was repeated but the animals received 1 % of squalene in the basal diet instead of a fixed daily quantity by the mouth; they were killed 24 hours after their last meal of the squalene-containing diet. As the results of the first experiment had shown that some of the substance was absorbed, no estimation of squalene in the faeces was made because it is very difficult to keep such estimations quantitative when dealing with any bulk of material; and with a substance of such highly unsaturated nature the results so obtained lose some of their significance when regard is paid to the high proportion of squalene excreted and the probability of oxidation occurring. In this experiment and the remainder of the work, it was therefore decided to confine attention to the livers of the animals only; and these were treated as described under Exp. 1.

Exp. 2. Duration of experiment, 36 days. Animals killed 24 hours after receiving their last meal of the squalene-containing diet.

Number of animals	6
Weight of livers, g.	50
Weight of unsaponifiable matter, g.	0.5424
Weight of cholesterol, g.	0.3114
Weight of residue not cholesterol, g.	0.2310
Weight of $C_{30}H_{50}6HCl$ obtained, g.	0.09

Experiments 3 and 4.

As the results of the first experiments had indicated that squalene was absorbed and that it appeared in the liver, it became of interest to carry out experiments with a view to determining whether it disappeared from the liver after the cessation of the administration of the substance to the rats, and these two experiments were carried out with that end in view. The animals received the diet containing 1 % of squalene and were killed at varying intervals after the squalene-containing diet had been replaced by the basal diet only.

Exp. 3. Duration of experiment, 17 days on a diet containing 1 % squalene. Animals killed at various times after their last meal on this diet, receiving meanwhile the basal diet only.

Time, hours		Weight of livers g.	Weight of unsaponifiable matter g.	Weight of cholesterol g.	Weight of material not cholesterol g.
—	Control	42	0.1451	0.1079	0.0372
6	„ + squalene	54	0.5344	0.3062	0.2282
15	„ + „	49	0.4774	0.2434	0.2340
26	„ + „	48	0.5812	0.3378	0.2434
50	„ + „	36	0.4064	0.2448	0.1616
50	„	50	0.2056	0.1463	0.0593

There was isolated 0.11, 0.10, 0.11, 0.07 g. respectively of $C_{30}H_{50}6HCl$ from the animals receiving the squalene diet. The control materials gave none of this hydrochloride.

Exp. 4. Repetition of Exp. 3. Duration of experiment, 17 days.

Time, hours		Weight of livers g.	Weight of unsaponifiable matter g.	Weight of cholesterol g.	Weight of material not cholesterol g.
18	Control	35.4	0.1316	0.0923	0.0393
18	„ + squalene	29.9	0.3012	0.1747	0.1265
66	„ + „	38.3	0.4388	0.2681	0.1707
186	„ + „	38.0	0.2072	—	—

DISCUSSION OF RESULTS.

As has been pointed out already, the first experiment shows that when squalene is administered to the rat, it is in part absorbed and as a result of this absorption there is a marked increase in the amounts of unsaponifiable matter and of cholesterol in the body and liver of the animal. Thus, even if the figure obtained for the weight of unsaponifiable matter in the bodies of the animals which received squalene be ignored because it is vitiated by the fact that they received their daily dose of squalene only a few hours before being killed, the big increase in cholesterol is significant. Thus, reducing the figures to the amount of cholesterol per kg. body-weight, we obtain for the control animals 1.058 g. and for those receiving squalene 1.539 g., an increase of about 50 %. A similar result holds for the livers in this experiment and, as the remainder of the work was carried out on the livers only, it will be convenient to consider all these results together.

The results are rendered clearer if a table be drawn up showing the weights of unsaponifiable matter, cholesterol and unsaponifiable matter not cholesterol, all the figures being calculated as grams per 100 g. of liver. The cholesterol is expressed as a percentage of the unsaponifiable matter.

		Control diet				Livers (100 g.).				Control diet+squalene			
Exp.	Hours	Un- saponifiable matter g.	Cholesterol g.	Un- saponifiable matter not cholesterol g.	Cholesterol %	Un- saponifiable matter g.	Cholesterol g.	Un- saponifiable matter not cholesterol g.	Cholesterol %	Un- saponifiable matter g.	Cholesterol g.	Un- saponifiable matter not cholesterol g.	Cholesterol %
1	3	0.5750	0.4025	0.1725	70.0	1.3830	0.7935	0.5895	57.4				
2	24	0.4092	0.3069	0.1022	75.0	1.0848	0.6244	0.4604	57.5				
3	6	0.3450	0.2697	0.0753	78.1	0.9895	0.5670	0.4225	57.3				
	15	—	—	—	—	0.9743	0.4967	0.4776	52.0				
	26	—	—	—	—	1.2108	0.7035	0.5073	58.1				
	50	0.4122	0.2926	0.1186	71.2	1.1297	0.6800	0.4497	60.2				
4	18	0.3717	—	—	—	1.0070	0.5843	0.4164	58.2				
	66	—	—	—	—	1.1457	0.7000	0.4457	61.1				
	186	—	—	—	—	0.6092	—	—	—				
Mean		0.4226	0.3179	0.1171	73.6	1.1156	0.6437	0.4711	57.7				

The amounts of squalene hexahydrochloride isolated from the livers of the animals which received squalene were 0.18, 0.18, 0.20, 0.20, 0.23 and 0.20 g. per 100 g. of liver in Exps. 1, 2 and 3, and 0.12 and 0.13 g. in the first two groups of Exp. 4. In calculating the mean values, the results obtained in the last group of Exp. 4 have been rejected as it would appear that the amount of unsaponifiable matter has reached a definitely lower figure and is not far removed from the average figure for animals fed on the basal diet only. The table shows that the unsaponifiable matter per 100 g. of liver of the animals

receiving squalene is 1.1156 g., as against 0.4226 g. in the livers of the control animals, an increase of 164 %. This increase is striking and coupled with it is an increase of just over 100 % in the amount of cholesterol, 0.6437 g. as against 0.3179 g., which results in there being a greater weight of cholesterol in the livers of the animals receiving squalene than there is total unsaponifiable matter in those of the control groups. It is to be noted that the increase in the non-cholesterol fraction of the unsaponifiable matter is not entirely accounted for by the presence in the livers of a corresponding amount of squalene, for although the hydrochloride of that substance was isolated from the livers, the amounts obtained were relatively small. Even allowing for the yield of this derivative being only 60 %, the actual amount of squalene present per 100 g. of liver would in no case exceed 0.2 g., whilst the average difference in the material not cholesterol is 0.3540 g.

It would be premature at this stage of the work to attempt to interpret this large increase of cholesterol in the livers of the animals receiving squalene with any certainty. Two possibilities arise—either squalene may be a precursor in cholesterol synthesis or else for some reason at present unknown the organ tends to adjust the ratio of cholesterol to other unsaponifiable matter present to as constant a value as possible. With regard to the first possibility, it is of interest to note that squalene, the molecule of which appears to consist of a long carbon chain containing six double bonds and branched methyl groups, yields a mixture of terpenes on distillation at atmospheric pressure, and Heilbron and Kamm [1926] comment on its possible relation to cholesterol. In this connection it is to be noted that although the present evidence points to it being normally absent from the liver, there seems to be present a substance which behaves in many ways like squalene [Channon and Marrian, 1926], and the fact that the animal body is capable of synthesising cholesterol [Channon, 1925] need not therefore negative the possibility of squalene being related to cholesterol.

The second suggestion as to the reason for the cholesterol increase would seem to receive some support from the fact that the increase in the unsaponifiable matter is not an increase in the cholesterol and squalene only, as already pointed out. It has been found in numerous experiments that the ratio of cholesterol to other unsaponifiable substances tends to a fixed value, as is shown for the figures obtained for the livers of the control animals in this experiment. The whole of these results may be due therefore to the absorption of squalene and its entry into the liver having caused an increase in cholesterol for some reasons of a physical nature. Further experiments are at present in progress to see whether an increase in the amount of the liver cholesterol can be caused by the administration of substances differing in chemical nature from squalene but having similar physical properties.

It will be seen from the results of Exps. 3 and 4 that there is no rapid return of the amount of unsaponifiable matter present in the liver to the normal value after depriving the animals of the squalene-containing diet, but the last

result obtained in Exp. 4 suggests that there is a distinct fall after a period of 8 days. In one experiment there seemed to be a sharp fall in the amount of unsaponifiable matter within 48 hours, but as all attempts to repeat this result have failed, it has been omitted from this paper. Further experiments are being carried out with a view to finding the rate of fall of the unsaponifiable matter to the normal value after the diet containing squalene is replaced by the basal diet.

No obvious ill effects followed the administration of squalene and *post mortem* examination showed that the organs of the animals were very healthy. There appeared to be a greater deposition of fat in the animals which received squalene and on the whole they seemed to grow at a somewhat greater rate than those of the control groups, possibly because the laxative action of the squalene caused a greater food consumption.

SUMMARY.

1. When the unsaturated hydrocarbon squalene (spinacene) $C_{30}H_{50}$ is included in the diet of the rat, some absorption occurs.
2. This absorption results in the amount of unsaponifiable matter in the liver increasing to about 2.6 times the normal.
3. The amount of cholesterol present increases by about 100 %.
4. Squalene can be isolated, as the hydrochloride, from the livers of the animals which have received it in their diet.
5. The increase in the amount of unsaponifiable matter in the liver is not wholly accounted for by the presence of squalene and the increase in cholesterol.
6. The possible significance of these results is discussed.
7. No ill effects followed the administration of squalene to the rat.

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