XXXVI. FURTHER STUDIES OF THE CHEMICAL NATURE OF VITAMIN A.

By JACK CECIL DRUMMOND and LESLIE CHARLES BAKER.

From the Department of Physiology and Biochemistry, University College, London.

(*Received February 26th, 1929.*)

Introduction.

In a previous paper on this subject [Drummond, Channon and Coward, 1925], the claim of Takahashi and his colleagues [1922, 1923, 1925] to have isolated vitamin A from cod-liver oil was criticised. It was shown that the fraction to which they gave the name "biosterin" was probably a complex mixture containing, in addition to the vitamin, unidentified unsaturated alcohols and possibly hydrocarbons of the squalene type. The investigation we are now reporting represents an extension of our earlier work, and the results provide conclusive proof that "biosterin" consists to a very large extent of substances other than vitamin A.

The earlier experience in this laboratory led us to believe that the considerable difficulties encountered in separating the constituents of the unsaponifiable fraction of cod-liver oil might to some extent be lessened if a much larger amount of material were available for fractionation than had formerly been employed. Accordingly plans were made for preparing the unsaponifiable fraction from a very large quantity of cod-liver oil. Through the generosity of Mr R. B. Job and Mr W. A. Munn of St Johns, Newfoundland, we were provided with 125 gallons of a high quality medicinal cod-liver oil. Actually the hopes based on the possession of the large amount of unsaponifiable material from this oil were not realised, for peculiar difficulties encountered in its examination made it advisable to change the line of attack.

It was decided to make a comparative study of the vitamin fractions from several different sources in order to ascertain whether a correlation between the vitamin content and any chemical characteristic could be traced. For this part of the investigation four materials were selected, namely, the unsaponifiable fractions from (*a*) cod-liver oil, (*b*) sheep-liver fat, (*c*) Greenland shark-liver oil, (*d*) Japanese shark-liver oil.

Although we have failed by this means to throw any clear light on the chemical nature of vitamin A, we believe we have established that the active substance is present in liver-oil concentrates in amounts so minute that direct attempts at its isolation by the ordinary chemical methods are of little use.

Experimental.

A. *Cod-liver oil.*

A large quantity of medicinal cod-liver oil from Newfoundland was saponified on a semi-technical scale in the laboratories of the British Drug Houses. We are greatly indebted to Mr F. H. Carr for his advice and assistance in carrying out this part of the work. The product yielded by this treatment was re-saponified in our laboratory with alcoholic KOH, and the extracted unsaponifiable fraction was again hydrolysed, this time with sodium ethoxide. All these operations were carried out with special precautions against oxidation.

The yield was about 0·7 % of the oil, and the product was a clean orange-coloured crystalline wax. The vitamin A colour value[1] was 13·3.

As in previous work, the first step was to remove the greater part of the cholesterol by crystallisation from methyl alcohol or light petroleum. In this manner several hundred grams of a dark red oil (I) with the following properties were obtained:

Cholesterol content	13·35 %
Refractive index $n_D^{40°}$	1·5022
Iodine value[2]	161
Vitamin A colour value	20

Our previous studies had led us to believe that this material contains in addition to cholesterol a number of unsaturated alcohols which might possibly be separated by the preparation of suitable derivatives. Weidemann [1926] attempted to separate the alcohols present in the corresponding fraction of Greenland shark-liver oil by means of the phthalates, but the record of his experience did not encourage us.

After a considerable amount of exploratory work along these lines we concluded that it would prove a matter of very great difficulty to effect any sort of separation of the constituents of the vitamin fraction of cod-liver oil by this means. No satisfactory crystalline derivatives were obtained, even

[1] Vitamin A colour values. It is not easy to find a satisfactory manner in which to express the relative powers of concentrates to yield the blue colour reaction of Rosenheim and Drummond. The most satisfactory estimations of the colour produced by arsenic or antimony trichloride under the conditions described by Carr and Price [1926] are made between 5 and 10 blue units. Throughout this paper the colour values of concentrates are expressed in the form of the reciprocal of the percentage concentration in grams which gives 10 blue units when 0·2 cc. of the chloroform solution is treated with 2 cc. of a 30 % solution of antimony trichloride in chloroform, and the colour measured after 30 seconds in a tube of 1 cm. diameter. We have relied almost entirely on the colour reaction for detecting and estimating vitamin A throughout this study. In an investigation which is shortly to be reported we have submitted the colour test to a further critical examination, and, as a result, believe it to be a more accurate method of estimating vitamin A than the biological method.

[2] The majority of the iodine values given in this paper were determined by using the method of Dam [1924].

when the substituted bromo- and nitro-phthalates were prepared, and finally we abandoned this line of attack.

There remained the possibility that with the larger amounts of material at our disposal a more satisfactory separation by distillation in high vacuum might be obtained. In some respects the distillations reported in the earlier work were unsatisfactory. In the first place they were conducted at relatively high pressures (1 to 2 mm.), and secondly, although it was shown by colour reaction and by feeding test that vitamin A had survived the process, no very clear evidence was obtained as to the amount of loss that had occurred. This was due to the unreliability of the biological test as a quantitative measure of vitamin A, and to the fact that the colour reaction was at that time (1924–5) carried out with sulphuric acid, and was therefore quite unsuitable for quantitative assay.

In view of the great expense we could not in the present study entertain the idea of removing completely by precipitation with digitonin the cholesterol present in our raw material, and an attempt was made to distil it without this treatment. This was done with some reluctance as we had always found more decomposition to occur when fractions were distilled without removal of the precipitable sterol.

The distillation was carried out in an apparatus so designed that it could be filled or swept through rapidly with a current of purified nitrogen. Nitrogen for this purpose was "scrubbed" satisfactorily by allowing the gas to escape from the cylinder through a fine-pored filter candle immersed in an alkaline solution of sodium hydrosulphite. It emerges in the form of a cloud of very small bubbles which do not coalesce, and a very satisfactory removal of the traces of oxygen which are normally present in cylinder nitrogen is attained, particularly if the column of hydrosulphite is high, and the gas is stored for a few hours in contact with the solution before use. The surfaces of the hydrosulphite in the washing vessel and the reservoir are protected by a layer of liquid paraffin.

The side arm of the distillation flask was fitted with a coil of nichrome wire, both inside and outside the receiver, so that electrical heating could be applied if there was any tendency for the distillate to thicken or solidify.

The maintenance of low pressures was assisted by having in the system a silica tube of adsorbent charcoal cooled in liquid air.

Distillation of red oil (I) from unsaponifiable matter of cod-liver oil.

As a preliminary experiment, 44 g. were distilled under what were regarded as satisfactory conditions, namely, uniformly low pressure of 0·01 mm. and a relatively small temperature difference (20–25°) between the metal-bath and the point of distillation. The course of the distillation is represented by curve *A* in Fig. 1, p. 288. It is unsatisfactory in that there is not much

evidence of fractionation. Either the material was a very complex mixture or decomposition had occurred.

The following fractions were, however, taken:

Fraction No.	Weight g.	Appearance	Temp.	Iodine value	Cholesterol %	$n^{40°}$	$\alpha^{20°}_{5461}$	Vitamin value
1	3·0	Pale brown liquid	80°–170°	119	0·0	1·4813	+0·8	0·14
2	1·7	Clear brown liquid, few crystals	170°–190°	115	2·12	1·4857	+0·5	0·86
3	4·8	Clear brown liquid	190°–222°	134	2·83	1·4872	−0·2	2·0
4	9·3	,,	222°–245°	176	10·80	1·4886	−2·7	0·5
5	6·8	Opaque brown semi-solid fat	245°–260°	176	20·65	1·4935	−6·95	0·22

The original material had a vitamin value of 20. If the recovery of vitamin be calculated from the weights of the fractions and their respective vitamin values it will be seen that it is as low as 2 %. Towards the end of the distillation the temperature was tending to rise rather rapidly, and signs of some decomposition were evident. A relatively large proportion of undistilled material (18 g.) remained behind in the flask in the form of a dark brown tar, which had an iodine value of 137, and contained 19·9 % of cholesterol.

All the fractions possessed the pungent terpene-like odour which suggested that decomposition had occurred. In our previous work [Drummond, Channon and Coward, 1925], we noticed that this odour was less pronounced when material was distilled after complete removal of the sterol. The estimations of cholesterol in the above fractions did not suggest that cholesterol itself decomposed, as the recovery was practically quantitative.

In spite of the very large loss of vitamin an attempt was made to study the material further. In view of the fact that no apparent separation had been effected by the distillation, the fractions that gave the best colour reactions (2, 3 and 4) were combined for further examination.

Some of this mixture was treated with digitonin to remove the precipitable sterol. It yielded a dark reddish brown oil with an iodine value of 138. As no crystalline products or derivatives could be isolated from it, an attempt was made to reduce it with hydrogen in the presence of a suitable catalyst in the hope that solid material would result. The oil proved very resistant to hydrogenation at room temperature when using as catalyst platinum prepared by the method of Feulgen [1921]. The hydrogen was absorbed very slowly, and it was found necessary to renew the catalyst several times. The hydrogenated product was a pale, brown-coloured, semi-solid fat possessing an iodine value of 48, and containing a small amount of glistening crystalline material. The solid portion was separated and recrystallised from methyl alcohol. It separated in the form of shimmering plates. Insufficient was obtained for satisfactory purification, but the best preparation melted at 54–56°. No other crystalline substance was isolated from the hydrogenated material. The colour reaction for vitamin A was not given by the reduced fraction.

The smoothly ascending distillation curve obtained in the distillation of the cod-liver oil unsaponifiable fractions, together with the unexpectedly great loss of vitamin A, led us to believe that more general decomposition had occurred during the heating than we had imagined. It was possible that decomposition products were responsible for the apparently rapid poisoning of the catalyst, and for the consequent slow uptake of hydrogen by the distilled fractions.

Accordingly, an attempt was made to reduce some of the undistilled red oil (I) after removing the sterol precipitable by digitonin. This material proved, however, to be equally resistant to reduction by hydrogen in the presence of platinum. In one experiment 10 g. dissolved in 200 cc. of absolute alcohol were treated with hydrogen in the presence of 0·1 g. of platinum. Hydrogen was admitted at a pressure of about an atmosphere and a half, and the reaction vessel rapidly shaken. The uptake of hydrogen was very slow, and several additions of fresh catalyst had to be made at intervals. Finally some 200 cc. of hydrogen were absorbed (calculated uptake 1220 cc.). From the reaction mixture 9·8 g. of a clear yellow oil were separated. On standing it deposited a crystalline material which was removed and recrystallised from methyl alcohol. Several fractions of this product, amounting in all to 0·17 g., were finally obtained in the form of white glistening plates. It melted at 67–69°, and appeared to be an incompletely purified hydrocarbon:

0·0176 g. gave 0·0544 g. CO_2 and 0·0330 g. H_2O.
Found: C, 84·28 %; H, 14·53 %.

The mother liquors from the crystalline hydrocarbon gave 8·9 g. of a clear red oil (II) with an iodine value of 118 and giving a fairly strong colour reaction for vitamin A. As no further crystalline material separated from this fraction after treatment with solvents it was distilled at 0·04 mm. Two fractions were obtained: (*a*) 2·05 g. boiling between 189–220°, I.V. 96, (*b*) 5·20 g. boiling between 220–270°, I.V. 138. Attempts were made to reduce these fractions more completely, as it was obvious that the original material had been very incompletely reduced, but they were unsuccessful, and only yielded small amounts of a product apparently identical with the impure hydrocarbon described above.

Our experience in carrying out the hydrogenation of the active fractions of cod-liver oil appears to have been in some respects similar to that of Nakamiya and Kawakami [1927]. The Japanese investigators found difficulty in carrying out a complete reduction when using palladium catalyst and temperatures up to 60°. From their treated products they isolated only small amounts of crystalline substances, accounting in all for some 5–10 % of the original "biosterin." The identification of some of their crystalline products is wholly unsatisfactory, but they claim to have detected nonocosane, batyl alcohol, melissyl alcohol and octadecyl palmitate. It is difficult to understand the presence of the latter substance in a material that is supposed to have

been completely saponified. The liquid fraction of their hydrogenated material was also a light brown oil, which did not solidify even at − 75°.

Further attempts to carry out a more satisfactory separation of the vitamin fraction by careful distillations were made. The great loss of vitamin A which we observed seemed to be in part caused by the incomplete removal of cholesterol and other sterols precipitated by digitonin. In the earlier studies [Drummond, Channon and Coward, 1925] material treated by digitonin was used, and the loss of vitamin was certainly much less, although we have no quantitative measure of the proportions. We could not afford to use digitonin on the scale necessary to treat large amounts of the red oil (I) from the unsaponifiable matter of cod-liver oil, and all efforts to distil it without such treatment were unsuccessful in that however carefully the distillation was conducted there was always a serious loss of the active substance and, we believe, considerable general decomposition. The failure of these efforts, which occupied many months, to separate the constituents of the unsaponifiable matter from cod-liver oil by distillation, or to isolate appreciable amounts of crystalline substances after reduction, led us to turn our attention to other rich sources of vitamin A.

Before describing this extension of the work, attention may be directed to a minor point of interest which arose during the cod-liver oil studies.

Presence of squalene in cod-liver oil.

In the earlier study by Drummond, Channon and Coward [1925] the presence in cod-liver oil of a hydrocarbon closely resembling squalene was described. On the basis of the yield of the crystalline hexahydrochloride and the octodecabromide it was estimated that the unsaponifiable fraction of the cod-liver oil used in that investigation—a Newfoundland medicinal oil—contained approximately 8 % of squalene.

During a study of the distribution of squalene in a large number of marine species, Channon [1928] failed to detect this hydrocarbon in the material extracted from authentic cod-livers or the livers of other *Teleostei*. He suggested that the presence of squalene in commercial cod-liver oils might be due to admixture with liver oils from fish other than gadoids. A similar view had been advanced by Nakamiya and Kawakami [1927] who failed to detect squalene in their "biosterin" fractions. During the past year we have been able to examine a number of cod-liver oils of unquestioned purity from various sources, and have in every case detected the presence of small amounts of squalene, or a similar substance, by the formation of the characteristic hexahydrochloride. In all cases the test was carried out by carefully preparing the unsaponifiable fraction and removing the greater part of the cholesterol by freezing out from dry methyl alcohol. After removal of the solvent the residue was dissolved in dry acetone and saturated with dry hydrochloric acid. If a crystalline precipitate was formed it was filtered off and washed well with dry ether to remove cholesterol hydrochloride. The

insoluble portion was recrystallised from dry acetone. The following results were obtained:

Oil			Unsaponifiable %	Hydrochloride mg. per 100 g. oil	Hydrochloride M.P.
Norwegian.	Spring	1928	0·66	12	123°
Aberdeen.	,,	1928	0·68	2·9	126°
Moray Firth.	,,	1928	0·65	8·0	126°
Newfoundland.	Summer	1927	0·67	3·5	126°
,,	,,	1928	0·67	3·2	125°
North Sea.	,,	1928	0·80	8·3	122°

These figures seem to establish that small amounts of squalene or a very similar hydrocarbon do occur in pure cod-liver oil.

The high iodine values of fractions 4 and 5 obtained in the distillation of the red oil (I) described above appeared to be due to the presence of this hydrocarbon, as comparatively large amounts of the characteristic hexahydrochlorides were isolated. From 1 g. of fraction 4 there was obtained a yield of 156 mg. of the hydrochloride, whereas the same amount of fraction 5 gave 218 mg. This product on recrystallisation showed a melting point of 126° and contained 33·7 % of chlorine (calculated for squalene hexahydrochloride 33·86 %).

B. *Sheep-liver fat.*

The unsaponifiable matter was prepared from a concentrate placed at our disposal by Mr F. H. Carr, by one treatment with alcoholic KOH and one with sodium ethoxide. It was a dark reddish brown, rather hard wax with a vitamin value of 20 and containing 35 % of cholesterol. The greater part of this constituent was removed by crystallisation from methyl alcohol, and a residual dark red oil (III) was obtained with an iodine value of 171 and a vitamin value of 35. A portion of this oil was distilled at a pressure of 0·012 mm. The distillation was very satisfactory as regards temperature control, and there were no apparent signs of decomposition. Nevertheless, a steep distillation curve (Curve *B*, Fig. 1) was obtained indicating that the mixture was either a very complex one or that decomposition had occurred.

The following fractions were, however, collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	$n^{40°}$	Vitamin value
1	2·7	Clear brown-yellow liquid	177°–190°	113	1·4861	6·7
2	2·0	Opaque brown liquid	190°–220°	149	1·4876	15·3
3	0·5	Semi-solid yellow mass	220°–230°	171	1·5043	8·3
4	2·8	Hard yellow wax	230°–260°	125	1·5089	3·7
5	2·7	Brownish yellow wax	260°–280°	138	1·5178	0·5

Of the original 16 g. of oil distilled only 10·7 g. were recovered in the fractions, and the residue in the flask was a thick black tar smelling strongly as if decomposition had occurred. Evidence of decomposition was also given by the very strong terpene-like smell of the distilled fractions.

Although some of the fractions showed fairly strong colour reactions it was apparent that the loss of vitamin A had been considerable from a calculation of the recovery (11 %) of chromogenic substance. Fractions 4 and 5 were combined and recrystallised from methyl alcohol. Nearly 1 g. of cholesterol was separated. The mother-liquors gave a deep red coloured oil (IV) from which no further crystalline material was obtained on treatment with various solvents. Fractions 1 and 2 were also combined and a small amount of cholesterol removed by precipitation with digitonin. The residual red oil (V) had an iodine value of 125, and was treated with hydrogen in the presence of palladium catalyst. It proved as resistant to reduction as the similar fractions from cod-liver oil, and after a long period in contact with the gas, and after several renewals of the catalyst, the iodine value had only fallen to 55.

It seemed possible that some of the difficulties encountered in the examination of this material might be due to the presence of the curious hydrocarbon found by Channon and Marrian [1926] in the liver fats of certain mammals. In order to test this possibility a small quantity of the fraction was tested by saturating the dry acetone solution with hydrochloric acid gas. A crop of a crystalline material was obtained, which differed in crystalline form from squalene hydrochloride and appeared to resemble the hydrochloride described by Channon and Marrian. A further 6 g. of the original unsaponifiable material (III) were freed completely from cholesterol, and fractionated from methyl alcohol in the manner described by these investigators. The following fractions were separated:

	I.V.
(*a*) 1·27 g.	123
(*b*) 0·3	134
(*c*) 0·11	154
(*d*) 0·30 (insoluble residue)	302

The iodine value of the corresponding fraction insoluble in methyl alcohol separated from pig-liver material by Channon and Marrian was 309. Bromination of our insoluble material gave 0·68 g. of the bromide similar in character to that described by them. This yield and an iodine value of 302 would correspond to the fraction containing about 81 % of the hydrocarbon with an iodine value of 370. A bromination of 0·5 g. of the original sterol-free unsaponifiable matter gave 0·275 g. of insoluble bromide. This corresponds with the material containing approximately 18 % of the hydrocarbon, and would allow an iodine value of about 80 for the other constituents.

The importance of the detection of such relatively large amounts of this curious hydrocarbon in the unsaponifiable fraction of sheep-liver fat lies in the observation of Channon and Marrian that it is readily decomposed when distilled under reduced pressure. A considerable production of liquids of low boiling point, together with a failure to obtain any bromide or hydrochloride from the distillates, seemed to indicate that the decomposition was extensive.

It is, we believe, decomposition of this type that is responsible for the loss of vitamin A by secondary reactions when the unsaponifiable material of cod-liver or sheep-liver oil is distilled under conditions that might be regarded as unlikely to cause destruction of the active substance.

An attempt was made to reduce with hydrogen and palladium the material more soluble in methyl alcohol obtained during the separation of the hydrocarbon. It was thought that the removal of the greater part of the hydrocarbon, itself of a constitution not readily reduced, might render the reduction of the residue more readily accomplished. Such did not prove to be the case. A solution of 1·79 g. dissolved in glacial acetic acid was treated with hydrogen under a pressure of about an atmosphere and a half. Hydrogen was very slowly absorbed, and several renewals of catalyst were made. In 5 hours 170 cc. of gas were absorbed (calculated 190 cc.), and the reduction seemed to have ceased. After hydrolysis of the reduced product, in case acetylation had occurred, 1·6 g. of a viscous golden yellow oil were obtained. This material failed to yield any crystalline product.

C. *Greenland shark-liver oil.*

The studies of Tsujimoto and Toyama [1922] and Weidemann [1926] suggested that the liver oil of the Greenland shark, *Somniosus microcephalus*, might yield an unsaponifiable fraction of simpler composition than the materials we have already described, and therefore more likely to provide information regarding the nature of the vitamin. The unsaponifiable fraction of this oil is known to consist to a large extent of cholesterol and the two dihydric alcohols, batyl alcohol and selachyl alcohol.

The sample of Greenland shark-liver oil employed in our work was a commercial preparation of dark orange colour, containing 15 % of unsaponifiable matter. From it 566 g. of this material were prepared. The iodine value of this fraction was 57, it contained 14·5 % of cholesterol and it possessed a colour index of 1·5. The relatively low vitamin value of this material did not deter us from examining its composition in view of the results we were obtaining at the same time in the examination of the vitamin-rich Japanese shark-liver oil (see Section D).

On attempting to separate the greater part of the cholesterol by washing with ice-cold methyl alcohol a large amount of insoluble pale yellow crystalline material separated. This was further washed with repeated changes of methyl alcohol at 0° and gave nearly one-third of the whole unsaponifiable fraction in the form of a white crystalline product. These crystals were obviously a mixture as they melted indefinitely between 65 and 100°. It appeared probable that in addition to the greater part of the cholesterol, the methyl alcohol had separated a solid alcohol of the type of batyl alcohol.

By repeated fractionation of a representative sample from light petroleum about half the expected amount of cholesterol was isolated in reasonably

pure condition, but as regards the other components only a number of fractions with melting points ranging from 60° to 90° were isolated. A few of these fractions were combined, and the cholesterol was removed by digitonin. The residue was a white solid, which, after several recrystallisations from methyl alcohol, melted at 68–69°. This appeared to be identical with batyl alcohol, M.P. 70–71°, and a mixed melting point determination with a sample of batyl alcohol, for which we are indebted to Prof. Tsujimoto, showed no depression.

A better method for the separation of the batyl alcohol and cholesterol, which together appear to make up practically the whole of that part of the unsaponifiable material insoluble in methyl alcohol, is to employ ether as a solvent. Batyl alcohol is sparingly soluble in cold ether and considerable amounts were separated in this manner. The purified product melted sharply at 69°:

Analysis. 0·1205 g. gave 0·3240 g. CO_2 and 0·1370 g. H_2O.

Found: C, 73·20; H, 12·62.

Calculated for $C_{21}H_{44}O_3$: C, 73·25; H, 12·79.

The fraction of the unsaponifiable matter soluble in methyl alcohol weighed 306 g., and was an orange coloured oil with a vitamin A colour value of 1·3.

Several very satisfactory distillations of this product were made. One of them is represented by curve *C*, Fig. 1, from which it may be seen that the chief part of the distillate is probably represented by one substance. The data regarding the fractions taken in this particular distillation of 45 g. are as follows:

Fraction No.	Wt. g.	Appearance	Temp.	Pressure mm.	Iodine value	Cholesterol %	$n^{40°}$
1	5	Yellow semi-solid mass	150°–235°	0·015	68·2	0·74	1·4578
2	7·9	Cream coloured semi-solid fat	240°	0·042	75·3	2·82	1·4631
3	5·4	Pale yellow liquid	240°–244°	0·045	81·4	3·4	1·4640
4	2·8	Clear brownish liquid	244°–250°	0·077	89·6	4·54	1·4623
5	9·7	„	250°–254°	0·10	91·2	3·98	1·4649

Of the 45 g. taken 26 g. boiled between 240–254° and appeared to be uniform, apart from a slight rise in iodine value as the distillation proceeded. The properties of these fractions suggested that they might be largely composed of unsaturated substances of the type of selachyl or oleyl alcohol.

	I.V.	$n^{40°}$
Selachyl alcohol	79	1·4691
Oleyl alcohol	94	1·4620

Very small amounts of a hydrocarbon, probably squalene, were detected by the formation of a crystalline hydrochloride.

2 g. of the undistilled fraction gave 18·7 mg. hydrochloride, equivalent, assuming a 50 % yield, to the presence of 0·6 % of squalene.

Only the first three fractions showed the colour reaction for vitamin A.

These were too pale to measure. 16 g. of the material, boiling between 240–254°, fractions 3, 4 and 5, were redistilled.

Fraction No.	Wt. g.	Temp.	Pressure mm.	Iodine value	$n^{40°}$
1	1·9	120°–130°	0·01	104·1	1·4561
2	4·2	150°–170°	„	87·6	1·4552
3	1·6	170°–180°	„	87·6	1·4570
4	2·8	180°–195°	„	84·6	1·4619
5	1·7	195°–203°	„	87·9	1·4722

Fractions 2–5 appeared to be uniform and were combined (fraction 134). 4·3 g. of the lower boiling material (fractions 1 and 2) from the main distillation were also refractionated. They yielded 2·7 g. of a semi-solid white wax boiling at 180–200° at 0·01 mm., with an iodine value of 74, and $n^{40°}$ 1·4630 (fraction 132).

Fraction 132. Although this material was semi-solid no satisfactory crystallisation from solvents was carried out, and it was subjected to hydrogenation. 2 g. dissolved in 100 cc. alcohol were treated with hydrogen and palladium catalyst. Absorption of hydrogen was rapid and a white crystalline material separated out in the reacting vessel during the later stages of the treatment.

The reduced substance (1·9 g.) was a white crystalline material melting at 53–54°. After recrystallisation from acetone, fractions of a white substance crystallising in clusters of very small, fine, curved needles were obtained, M.P. 60–61°. This material was later combined with similar fractions obtained by the reduction of fraction 134.

Fraction 134. Cholesterol was removed quantitatively from this material by digitonin, leaving a brown oil; I.V. 86.

It was reduced with hydrogen and palladium very readily, and yielded a white crystalline product melting at 45–50°. After two recrystallisations from acetone it melted at 65–66°.

Fractionation of crystalline material from hardened fractions 132 *and* 134.

As it appeared likely that the products of hydrogenation of fractions 132 and 134 were very similar in composition they were combined for a systematic fractionation from acetone. The less soluble fractions all with melting points above 59° were united and recrystallised from methyl alcohol. The melting point of the product was 64–65°.

Analysis. 0·0722 g. gave 0·1942 g. CO_2 and 0·0812 g. H_2O.

Found: C, 73·35 %; H, 12·63 %.

Calculated for $C_{21}H_{44}O_3$: C, 73·25 %; H, 12·79 %.

The phenylurethane melted at 96–97·5°. This product appeared, therefore, to be batyl alcohol. Heilbron and Owens [1928] give the melting point of the urethane as 98°. The fractions more soluble in acetone, with

melting points below 59°, were combined and 2·75 g. were redistilled at 0·06 mm.

Fraction No.	Wt. g.	Temp.
1	0·5	140°–155°
2	0·65	155°–191°
3	0·55	190°–200°
4 Residue	0·7	—

Each fraction was recrystallised from methyl alcohol. The melting points of the recrystallised materials were (1) 51·8°, (2) 54·5°, (3) 56·6°, (4) 57·5°.

Fractions 1 and 2 were analysed:

(1) 0·0768 g. gave 0·2190 g. CO_2 and 0·0930 g. H_2O.
Found: C, 77·76 %; H, 13·45 %.

(2) 0·0762 g. gave 0·2157 g. CO_2 and 0·0904 g. H_2O.
Found: C, 77·20 %; H, 13·18 %.

Calculated for octadecyl alcohol ($C_{18}H_{38}O$): C, 79·91; H, 14·17.
Calculated for batyl alcohol ($C_{21}H_{44}O_3$): C, 73·25; H, 12·79.

The phenylurethanes were prepared, and melted at 71–73°. Heilbron and Owens [1928] give the melting point of the phenylurethane of octadecyl alcohol as 77–78°; André and Francis [1926] give 79–80°. Attempts were made to purify the parent substances, but it was found to be a very difficult matter. The material isolated from these hardened fractions behaved very much like a mixture of batyl and octadecyl alcohols, when compared with mixtures of known composition of the two substances. Prof. Heilbron informs us that he has encountered the same difficulty in separating mixtures of the urethanes of these two substances.

From these studies we concluded that the unsaponifiable matter from the liver oil of the Greenland shark consists to a large extent of cholesterol, batyl alcohol and unsaturated alcohols—probably selachyl and oleyl—which on reduction yield respectively batyl and octadecyl alcohols.

D. *Japanese shark-liver oil.*

Through the courtesy of Mr John Spencer of Aberdeen we were able to obtain a sample of commercial Japanese shark-liver oil of relatively high vitamin value. This oil contained approximately 5·5 % of unsaponifiable matter and gave a vitamin test of 0·2. The unsaponifiable fraction was a clear pale yellow oily fluid, I.V. 78, and on standing a slight deposit of crystalline material separated. Small amounts of cholesterol were found. 160 g. of this unsaponifiable material were recrystallised from methyl alcohol and yielded 34 g. of crystalline substance. The melting point of this product, after many recrystallisations from light petroleum and ether, rose to 62–62·5°. It crystallised in sheaves of needles from ether, in which it is not very soluble. It appeared to be identical with chimyl alcohol, $C_{19}H_{40}O_3$, described by Toyama [1924], M.P. 60·5–61·5°.

Analysis. (1) 0·0707 g. gave 0·1880 g. CO_2 and 0·0821 g. H_2O.
Found: C, 72·51 %; H, 12·9 %.
(2) 0·0653 g. gave 0·1725 g. CO_2 and 0·0747 g. H_2O.
Found: C, 72·03 %; H, 12·71 %.
Calculated for $C_{19}H_{40}O_3$: C, 72·14 %; H, 12·66 %.

The phenylurethane crystallised in long needles from ethyl alcohol, M.P. 98°.

Treatment of chimyl alcohol with hydriodic acid. In order to isolate and identify the fatty alcohol present in the chimyl alcohol we reduced it with hydriodic acid in a micro-Zeisel apparatus so that we were enabled to make an estimation of the glycerol as *iso*propyl iodide at the same time. The reduction was carried out in the following manner. The reaction flask was heated slowly to 110° and maintained at that temperature for half an hour. The temperature was then gradually raised to 130° for a further 30 mins., and finally to 145–150° for 10 mins. Under these conditions we obtained yields of silver iodide corresponding to 23·19, 23·85 and 23·15 % glycerol. The theoretical value for chimyl alcohol is 29·12 %. We have not succeeded in obtaining values nearer the theoretical than those given above, although we have attempted to modify the conditions of the reaction so as to render smaller the likelihood of secondary reactions occurring. Parallel determinations on pure samples of batyl alcohol gave values of 21·5, 21·97 and 22·02 % as compared with the calculated figure of 26·74 %.

After the treatment with hydriodic acid the contents of the flask in the case of the experiments with chimyl alcohol contained an oily layer, whereas in the case of batyl alcohol the layer solidified to a soft wax. The oily layer in the former case was extracted with ether, and washed with sodium bisulphite and with sodium bicarbonate solutions. The crude iodide was recrystallised twice from acetone and melted at 23–25°. This product appeared to be cetyl iodide, M.P. 23° [Gascard, 1921]. Some of the iodide was converted into the corresponding alcohol by treatment with sodium acetate and subsequent saponification. The cetyl alcohol after recrystallisation from acetone melted at 44–47°.

These observations serve to confirm the constitution of a monoglyceryl ether of cetyl alcohol for chimyl alcohol suggested by Heilbron and Owens [1928] as a result of their studies, which revealed batyl alcohol to be an ether of octadecyl alcohol and glycerol.

The fraction of the unsaponifiable matter soluble in methyl alcohol deposited on standing a considerable amount of additional crystalline material which was separated by centrifuging. Both solid and liquid fractions were treated with digitonin to remove a small amount of cholesterol. The liquid oil after this treatment possessed an iodine value of 103 and a vitamin value of 3·75.

35 g. of this oil were distilled at 0·04 mm., and the following fractions collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	$n_D^{20°}$	Vitamin value
1	1·7	Yellow-brown liquid, some crystals	120°–190°	69	1·4720	3·3
2	2·6	,,	190°–202°	84	1·4762	4·0
3	8·2	Yellow semi-solid mass	202°–205°	100	1·4755	3·3
4	7·9	,,	205°–206°	101	1·4731	1·4
5	8·1	,,	206°–208°	101	1·4725	0·6
6	4·2	Yellow wax	208°–210°	103	1·4725	0·4
7	3·3	Hard yellow wax	210°–220°	107	1·4769	1·0

As shown by the distillation curve *D* (Fig. 1) this material seems to be uniform, and the results of the examination of the fractions confirm this view. The recovery of vitamin was nearly 50 % and in fraction 2 there was actually a slight concentration of the active substance. As regards the vitamin this was the most successful distillation we have had in the course of 5 years' work. Fractions 3 and 4 were reduced with hydrogen in the presence of palladium at room temperature, the absorption of hydrogen being very rapid. From both fractions a white crystalline material was isolated in quantitative yield. Even in the crude condition it appeared to be fairly pure batyl alcohol, and on recrystallisation from ether this substance was obtained readily, M.P. 67–69°; phenylurethane, M.P. 98·5–99°. The small amounts of material in the mother-liquors from the purification of the batyl alcohol were soft waxes with iodine values of about 74. They contained batyl alcohol, but there was insufficient to isolate the other constituents. They did not give a colour reaction for vitamin A. Small traces of the hydrocarbon like squalene were detected in the distilled fractions, but it is doubtful whether they were sufficient to account for the iodine values of 102–107 on the assumption that the main constituent was selachyl alcohol (I.V. 79).

The solid material that deposited on standing from the methyl alcohol-soluble fraction of the unsaponifiable matter was also distilled at 0·01 mm. after removal of the cholesterol by digitonin. It had an iodine value of 90 and a vitamin value of 3·1. The following fractions were collected:

Fraction No.	Wt. g.	Appearance	Temp.	Iodine value	$n_D^{20°}$	Vitamin value
1	0·9	Yellow-brown semi-solid wax	90°–180°	61	—	1·2
2	4·4	,,	180°–195°	81	1·4790	1·9
3	9·4	,,	195°–204°	87	1·4788	0·7
4	10·8	Pale yellow wax	204°–208°	90	1·4778	0·3
5	2·2	Brown wax	208°–227°	100	1·4755	0·6

The distillation curve is shown in curve *E*, Fig. 1, and is very similar to that of the corresponding liquid fraction (curve *D*). The recovery of vitamin is not so good, 23 % as compared with 48 %, but in this case there was some preliminary manipulation which may slightly have damaged the product. The information regarding the fractions again suggests that mainly one substance is present, and confirmation of this was obtained from the reduction

experiments which yielded almost quantitative amounts of batyl alcohol, M.P. 68–69°; phenylurethane, M.P. 98·5°.

It would seem, therefore, that the greater part of the fraction of the unsaponifiable matter soluble in methyl alcohol is selachyl alcohol. (Selachyl alcohol, B.P. (5 mm.) 236–239°, I.V. 79, $n^{20°}$ 1·4691.) An analysis was made of fraction 4:

(*a*) 0·0832 g. gave 0·2262 g. CO_2 and 0·0925 g. H_2O.
Found: C, 74·14 %; H, 12·28 %.

(*b*) 0·0806 g. gave 0·2192 g. CO_2 and 0·0893 g. H_2O.
Found: C, 74·16 %; H, 12·31 %.

Calculated for $C_{21}H_{42}O_3$: C, 73·69 %; H, 12·28 %.

Determinations of glycerol on the crude hydrogenated fractions (M.P. 58–60°) gave 22·24 and 22·11 %, figures agreeing very well with those obtained on pure batyl alcohol.

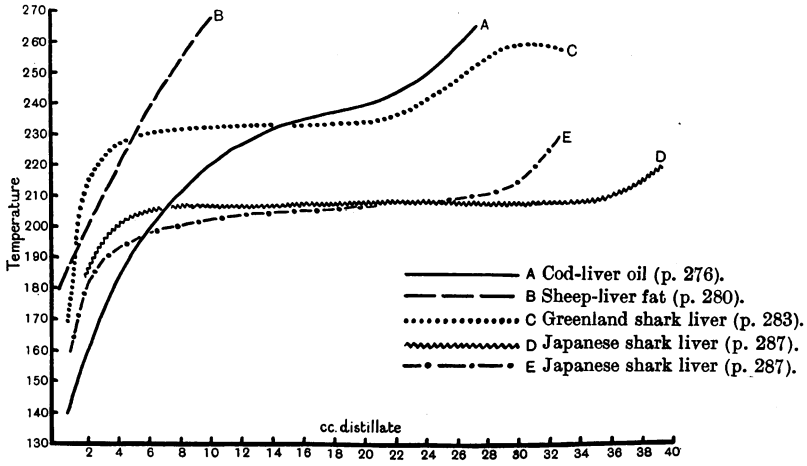

Fig. 1. Distillation curves of unsaponifiable fractions.

Only the fact that most of the fractions were more or less solid waxes was against their being almost wholly composed of selachyl alcohol. Pure selachyl alcohol freezes at about 5–10°, but it remains a liquid at temperatures at which our fractions were solid. It is possible that the substance present in the material we were examining was an isomer of selachyl alcohol. We are investigating this matter further.

Discussion.

Viewed from the standpoint of the main object of the investigation, namely, to ascertain the chemical nature of vitamin A, the observations recorded in this paper are unimportant. They serve, however, to make it clear that the task of isolating this vitamin is one of even greater difficulty

than our previous experience had led us to believe. The results obtained in the study of the Japanese shark-liver oil are sufficient to show that the vitamin forms a very minute proportion of the total unsaponifiable matter. The original oil possessed a vitamin A value comparable with that of a very good cod-liver oil. From the unsaponifiable fraction were isolated, either directly or after reduction with hydrogen, recognisable substances in reasonably pure condition in amounts accounting for 90–95 % of the total material. As far as could be ascertained the residue consisted to a large extent of these same substances in less pure condition.

It is generally recognised that the separation of the components of mixtures of fatty substances of low melting points is in all cases exceedingly difficult, and that it is well-nigh impossible if the desired substance represents a small proportion of the mixture. After removal of the cholesterol and chimyl alcohol from the unsaponifiable fraction of the Japanese shark-liver oil a residue was obtained which could be regarded as slightly impure selachyl alcohol.

It distilled completely over a narrow range of temperature, and on treatment with hydrogen yielded almost quantitatively the corresponding saturated compound, batyl alcohol. We are forced, therefore, to conclude that the vitamin is present in amounts so small that its separation will not be attained by such methods as we have used, unless it be found that a characteristic derivative can be prepared which possesses properties suitable for its isolation.

In illustration of this view there can be given the case of the hydrocarbon (squalene) that was found in some of the fractions. Fractions 4–6 of the distillation described on p. 287 appeared to consist almost entirely of selachyl alcohol, but possessed an iodine value (100–103) which suggested that a small amount of an impurity with a much higher degree of unsaturation than that substance was also present. Owing to the fortunate fact that very small amounts of this hydrocarbon can be detected by the formation of the insoluble hydrochloride or bromide it was possible to demonstrate that some, if not all, of the excess of the iodine value of these fractions over that of selachyl alcohol could be accounted for in this manner. Only 7 % of squalene would be required to raise the iodine number of these fractions from 79 (selachyl alcohol) to the observed value of 100, and yet, were the separation of the bromide and hydrochloride not an easy matter, the detection, much more the isolation, of this constituent would be an exceedingly difficult task.

The results reported in this paper throw no definite light on the chemical nature of vitamin A, nor do they provide reasonable grounds for speculation. So far only one indication has been obtained, and it is one that we think points the direction in which work might profitably be directed in the future. The colour reactions believed by many to be specific for vitamin A recall so strongly those given by certain types of sterol derivatives, that it is probable that more progress in elucidating the nature of the active substance will be made by studying the properties of sterols than by continuing to employ such

methods as we have used during the last ten years. It is encouraging to recall the brilliant success which has followed the studies of the sterols in relation to vitamin D, and to remember that the concentrated preparations of the antirachitic vitamin that are now available would probably never have been obtained by employing such methods as we have used in attempting the isolation of vitamin A.

Summary.

1. Further attempts have been made to separate by fractional distillation at pressures of about 0·01–2 mm. the vitamin A present in the unsaponifiable matter of certain liver oils. These efforts have not succeeded.

2. The unsaponifiable fraction of cod-liver oil, after removal of the greater part of the cholesterol, does not fractionate satisfactorily, but tends to decompose with rather serious loss of the vitamin.

3. The constituents of the unsaponifiable fraction of cod-liver oil are not readily reduced by hydrogen in the presence of platinum or palladium catalysts, and little information regarding their nature was obtained by this line of attack. It was also found impracticable to effect their separation by the preparation of phthalates or substituted phthalates.

4. The unsaponifiable fraction from sheep-liver fat also decomposed considerably on distillation in a high vacuum. Part of this decomposition, which involves the vitamin, is due to the presence of the highly unsaturated hydrocarbon, resembling in some respects squalene, discovered by Channon and Marrian in mammalian livers. Considerable amounts of this substance were separated from the vitamin fractions.

5. The sheep-liver fractions were as resistant to hydrogenation as those from cod-liver oil.

6. The unsaponifiable fractions of Greenland shark-liver oil and Japanese shark-liver oil consist largely, as the studies of Tsujimoto and Toyama have indicated, of selachyl, batyl, chimyl and oleyl alcohols.

7. The distillation of the unsaponifiable fractions from these oils is accompanied by comparatively little destruction of the vitamin, owing, it is thought, to the small proportion, or absence, of the complex alcohols and hydrocarbons of the terpene series.

8. A sample of Japanese shark-liver oil possessing a vitamin activity of the same order as a good cod-liver oil yielded an unsaponifiable fraction of which 90–95 % was accounted for in the form of reasonably pure preparations of the alcohols mentioned in 6, together with small amounts of cholesterol and a hydrocarbon resembling squalene. The remainder appeared to consist to a large extent of the same substances. This would indicate that the vitamin A forms a very small proportion of the unsaponifiable matter, probably less than 1 %, and supports the view expressed formerly by us that the "biosterin" of Takahashi and his colleagues is an extremely crude preparation of the active substance.

9. The structure suggested by Heilbron and Owens [1928] for chimyl alcohol, namely, that of a monoglyceryl ether of cetyl alcohol, has been confirmed by using methods similar to those employed by them in determining the constitution of the related batyl alcohol.

We are greatly indebted to the Medical Research Council for a series of grants during the past few years that have defrayed the cost of this investigation.

REFERENCES.

André and Francis (1926). *Compt. Rend. Acad. Sci.* **183**, 663.
Carr and Price (1926). *Biochem. J.* **20**, 497.
Channon (1928). *Biochem. J.* **22**, 51.
—— and Marrian (1926). *Biochem. J.* **20**, 409.
Dam (1924). *Biochem. Z.* **152**, 101.
Drummond, Channon and Coward (1925). *Biochem. J.* **19**, 1047.
Feulgen (1921). *Ber. deutsch. chem. Ges.* **54**, 360.
Gascard (1921). *Ann. Chim.* (9), **15**, 373.
Heilbron and Owens (1928). *J. Chem. Soc.* 942.
Nakamiya and Kawakami (1927). *Sci. Papers, Inst. Phys. Chem. Res.* **7**, 121.
Takahashi (1922). *J. Chem. Soc. Japan*, **43**, 826.
—— and Kawakami (1923). *J. Chem. Soc. Japan*, **44**, 590.
—— Nakamiya, Kawakami and Kitasato (1925). *Sci. Papers, Inst. Phys. Chem. Res.* **3**, 81.
Toyama (1924). *Chem. Umschau*, **31**, 61.
Tsujimoto and Toyama (1922). *Chem. Umschau*, **29**, 27, 35, 43.
Weidemann (1926). *Biochem. J.* **20**, 685.