CLI. STUDIES ON THE CHEMICAL NATURE OF VITAMIN A.

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Efforts to isolate and to determine the nature of the substance known as vitamin A received a great impetus when it was definitely established that the compound may be concentrated into that part of such substances as cod-liver oil which resists saponification [Steenbock and Boutwell, 1920; Drummond and Coward, 1921][1]. Owing to the instability of the vitamin in the presence of substances tending to produce oxidation it is usually necessary, if loss is to be prevented, to carry out the saponification and extraction in an atmosphere of an inert gas such as nitrogen. When this precaution is taken, it is a comparatively simple matter to concentrate with little or no appreciable loss the vitamin A present in a litre of cod-liver oil into the 8 or 9 g. of unsaponifiable matter that this yields. Furthermore it has been demonstrated that this preparation also contains the whole of the anti-rachitic vitamin, or vitamin D, of the raw oil [Zucker, Pappenheimer and Barnett, 1922; Zucker, 1922]. The value of concentrating these physiologically important substances may be appreciated from two standpoints. In the first place it is obvious that there is a definite, if limited, need in clinical medicine for a preparation containing in highly concentrated form the components of the oil that are responsible for its therapeutic action; indeed, at least two patents have been taken out and are being operated for the technical preparation of such material for medicinal use. On the other hand the possession of a concentrated fraction, obtainable in reasonably large amounts, provides some encouragement for the attempt to isolate the active constituents.

During the last five years we have been concentrating our efforts in this field, without, we regret to say, any success worthy of note. Our reasons for

[1] As far back as 1914 McCollum and Davis reported that the growth-promoting vitamin in butter fat withstood saponification [1914]; although later work has shown this to be true, the results of their experiments could not be claimed as being conclusive evidence on this point.

publishing what is admittedly an incomplete study are two: in the first place, we feel that unless very much larger quantities of raw material (unsaponifiable matter) are available than we have hitherto possessed, the technique by which we have attacked the problem in the past is quite inadequate to carry us much further than we have already gone. Secondly, we desire to give our reasons for challenging a number of claims that have recently been advanced, in particular those of Takahashi [1922, 1923, 1925] and his colleagues, to have isolated vitamin A in pure condition.

Preparation of the unsaponifiable matter for the study of vitamin A.

The preparation of the concentrate which has been used by us as the raw material for our fractionations scarcely needs full description now since several processes have been given in detail, either in papers or patent specifications, by other investigators. Several points may, however, be emphasised as possibly being of help to others wishing to repeat this work. In these studies simple saponification of the cod-liver oil with potassium hydroxide in alcohol has been exclusively employed. As a second saponification is always necessary it is preferable to make the initial treatment with alkali as short as possible, in order to minimise both inactivation of the vitamin and formation of resinous material from the reagents. The oil is slowly poured with vigorous stirring into the hot solution of the alkali in alcohol; a 10 % excess over the theoretical quantity of potassium hydroxide being ample. When all the oil[1] has been added, the mixture is heated to boiling for about 30 minutes, cooled, diluted with 10 volumes of water, and the unsaponifiable matter extracted with ether. An examination of the product yielded by this initial saponification reveals the fact that practically always a considerable proportion of matter still present can be hydrolysed. It is therefore advisable to submit it to a second saponification, for which it has been found preferable to employ sodium ethylate. It is important that a stream of nitrogen should be passed through the apparatus while this saponification is in progress, for it has been our experience to find that considerable loss of vitamin A may take place at this stage of its extraction, unless great care be taken to prevent contact with air. It is scarcely ever necessary to resort to a third treatment with alkali, the action of the sodium ethylate being sufficient not only to resolve the glycerides which have survived the initial process, but to hydrolyse the fatty acid esters of cholesterol and other higher alcohols present.

The importance of obtaining a product entirely free from esters is considerable if it is desired to submit it to fractional distillation, for we have

[1] In our earlier investigations we employed a pale Norwegian oil of high technical quality. Later, as the result of other investigations [Zilva and Drummond, 1923], we found that the vitamin A value of oils from Newfoundland is usually many times greater than that of Norwegian oils from the Lofoten area, and, accordingly, we employed as far as possible oils from the Dominion as our raw material. We are indebted to Mr W. A. Munn and Mr Davies of St John's, Newfoundland, for supplies of oils from this source.

observed that the presence of small amounts of such substances tends to give rise to more decomposition during the distillation at low pressures than is desirable. No material was employed by us for further treatment until a test carried out on a small sample showed that saponification had been complete. There appears no doubt that the method of saponification employed by Zucker [1922], and by Takahashi [1922], in which a large proportion of the fatty acids are removed in the form of the relatively insoluble calcium or barium soaps is more advantageous than the rather tedious process we adopted, although the yield of unsaponifiable matter appears to be slightly larger in our case. We have found ethyl ether to be the most convenient solvent for the extraction of the unsaponifiable matter from the alkaline solutions of the soaps, but its suitability as an agent of extraction is lessened to some extent by the difficulty of obtaining it pure in large quantities. The problem of extracting the active material from the products of hydrolysis of some hundredweights of cod-liver oil is not an easy one, even when the laboratory is equipped with small scale "plant," and the choice of an extracting agent is particularly difficult. The employment of ether may lead to a variety of troubles, of which the accumulation of the less volatile impurities usually present in the solvent —mainly mixed ketones of the type of methylpropyl ketone—in the final product is less disturbing than loss of material and damage caused by an explosion due to the accumulation of so-called "ether peroxide." Some experience of these troubles has led us to investigate a number of other solvents, particularly the inert hydrocarbons, but the latter tend to give rise to troublesome emulsions, and we have not yet found a solvent satisfying our requirements. Meanwhile we have continued to employ ether. The yield of material obtained from the initial hydrolysis of ordinary samples of cod-liver oil is usually from 1·5–2 %, and this is reduced by further saponification to about 0·8 %. The product is a semi-solid crystalline mass with a waxy appearance and varying in colour from pale golden yellow to deep orange according to the colour of the original oil. A brown or brownish red colour usually indicates that the production of resin during the saponification has been unnecessarily great. The crystalline appearance can readily be recognised as being due to flat prisms of cholesterol. Tested for the presence of vitamin A either by feeding tests with rats or by means of the colour reactions recently described [Rosenheim and Drummond, 1925] this preparation, if made and stored with due precautions against oxidation, is found to be equivalent to the oil from which it was extracted.

Chemical examination of the unsaponifiable fraction of cod-liver oil.

Iodine and Nitrogen.

An examination of the unsaponifiable matter revealed that the constituents were entirely organic in nature, and that the only recognisable elements

present were carbon, hydrogen, and oxygen. Very careful tests were made to detect any trace of iodine or nitrogen, because of the still lingering theories that these elements are connected with the physiological activity of the oil. Nearly a century ago Hupfer de l'Orme [1836] first detected the presence of iodine in cod-liver oil and suggested that the therapeutic action was due to traces of that element. At first the theory was coldly received and even the presence of iodine was doubted [Gmelin, 1837; Potemka, Martius and Sarphati, 1838], but when it had been established definitely that this halogen was usually present in the oils then on the market [Springmüll, 1837; Gmelin, 1838; Bennett, 1841], the theory became widely accepted. Indeed it was almost generally held until Williams [1912, 1913] pointed out that the therapeutic action of cod-liver oil is unrelated to its iodine content and suggested as an alternative theory that it is probably due to the readiness with which the highly unsaturated fatty acids present in cod-liver oil might conceivably be assimilated by the organism. Until recent years, the fatty acid theory and the iodine theory may be said to have shared popularity. The demonstration that the therapeutic action of cod-liver oil can be transferred to the unsaponifiable fraction and that the fatty acids themselves are without action [Drummond, 1919] rendered the former hypothesis no longer tenable. The iodine theory survived, however, because it was possible that the active complex containing iodine might be concentrated into the unsaponifiable matter, until it was shown that there are no detectable traces of the halogens in the active fractions [Drummond and Coward, 1922]. This we have many times confirmed ourselves, and it has been established also by an independent test kindly carried out for us by Dr Harington on a relatively large quantity of a highly active product. In view of these observations, as well as of the fact that the medicinal value of cod-liver oil cannot, as far as our experience goes, be correlated with its iodine content, we feel that there are no adequate reasons for the recent suggestions that it may be necessary to revive the iodine theory. The absence of all traces of nitrogen from the unsaponifiable fraction, when carefully prepared, demonstrates that the substances responsible for the therapeutic action of the cod-liver oil do not contain this element. The work of Funk [1915, 1916] in which processes are described for the isolation of vitamins from cod-liver oil, may be dismissed because the methods could only be concerned with the extraction of substances of the type of nitrogenous bases. This investigator must however, be given credit for being the first to recognise that the curative value of cod-liver oil in rickets and wasting diseases is probably due to one of the substances then so recently discovered by Hopkins [Funk, 1914].

Cholesterol.

As far back as 1886 Salkowski recognised the presence of cholesterol and of a lipochrome, not carotene, in the unsaponifiable fraction of cod-liver oil [1886]. He proved the identity of the cholesterol satisfactorily and showed

that the slight laevo-rotation exhibited by cod-liver oils can be accounted for by the presence of this substance. His observations have been confirmed by us. It has been shown previously [Drummond and Coward, 1922] that the cholesterol may be quantitatively removed by crystallisation and precipitation with digitonin without removing vitamin A, and this observation has been confirmed many times by ourselves and by other observers [Takahashi and Kawakami, 1923].

Removal of Cholesterol.

Estimations of cholesterol by the digitonin method in a large number of samples of unsaponifiable matter from cod-liver oil give values ranging from 47·8 to 50·9 %. The majority of values are close to 50 %. The removal of this constituent is best effected in our opinion by the following process. The weighed material is treated with twice its weight of boiling methyl alcohol, nitrogen being bubbled through the solution, and the orange yellow liquid is then cooled in ice. Approximately 90 % of the cholesterol present separates and may be removed by filtration, preferably conducted in an atmosphere of nitrogen. After repeated crystallisation from alcohol, acetone and light petroleum, the cholesterol shows M.P. 148·5–149° (corr.), and gives acetate, M.P. 114·3°, benzoate, M.P. 145°; and has $[\alpha]_F^{15°}$, − 40°. It is inadvisable to cool the liquid much below zero in separating the cholesterol because other constituents tend to crystallise out at lower temperatures. The removal of the residual cholesterol can only be satisfactorily carried out by the method of precipitation with digitonin introduced by Windaus. The great expense of this reagent and the difficulty of obtaining it in quantity have proved the main factors limiting our studies. The mother liquors from the crystallisation of the cholesterol are evaporated at low pressure to remove the methyl alcohol, and any further cholesterol separating during the concentration is removed; if the cooling of the original solution has been prolonged, there is usually little or no further separation. For the preparation of material for therapeutic use, it is, of course, unnecessary to remove the cholesterol entirely, and the examination of several products at present on the market has shown that only the preliminary separation by crystallisation from a suitable solvent has been employed. Such preparations usually contain from 5 to 9 % cholesterol. The removal of the remaining cholesterol is carried out by dissolving the material in four volumes of 95 % alcohol and adding the necessary excess of a 1 % solution of crystalline digitonin (Merck) in boiling 90 % alcohol; the solution is maintained just below boiling point while a slow current of nitrogen is passed continuously through it. After tests have shown that the precipitation of the cholesterol digitonide is complete, the mixture is allowed to stand overnight at room temperature, and the digitonide is removed on the following morning by filtration. The precipitate must be thoroughly washed with 95 % alcohol and finally with a little ether. The filtrate with the added washings is evaporated at low temperature and pressure to remove

the solvents, the residue dissolved in ether, and the small excess of digitonin washed out by repeatedly shaking the ethereal solution with water in an atmosphere of nitrogen. Finally the ethereal solution is separated and dried with anhydrous sodium sulphate and the solvent removed at low pressure. The product thus obtained is a deep reddish orange oil possessing the characteristic smell of the original unsaponifiable fraction. If it is carefully prepared it shows a vitamin A value proportionate to the cod-liver oil from which it was prepared. The cholesterol, whether prepared by purification of the material separating from the methyl alcohol solution, or from the digitonide by decomposition with hot xylene vapour in an inert atmosphere, shows no physiological activity. This confirms our earlier observations on the inactivity of pure cholesterol from other sources, *e.g.* brain [Drummond, 1919].

Fractionation of the Cholesterol-free Residue.

A superficial examination of the viscous oil which remained after cholesterol had been removed suggested that it was to a large extent composed of substances of the nature of unsaturated alcohols. Accordingly, efforts were made to separate the constituents by means of fractional distillation. A brief outline of our earlier results was communicated to the Biochemical Society at the meeting on May 10th, 1924 [Drummond and Coward, 1924], in which it was shown that vitamin A is volatile in superheated steam at 115–125°, and that by fractional distillation at low pressure it could be concentrated in a fraction boiling at about 250° (3–4 mm.).

Distillation in Superheated Steam.

As a method of effecting any further concentration of vitamin A we soon abandoned this method, for not only is it very tedious, but it effects no separation of the constituents other than the removal of the non-volatile resins derived from the saponification. The distillation must be carried out in an atmosphere of nitrogen a rapid stream of which must sweep through the apparatus before distillation commences, as well as throughout the experiment. The superheated steam slowly carries over the volatile constituents of the oil, the distillate condensing as an emulsion of semi-solid consistency. To avoid temporary blocking of tubes by the firm candle-like emulsion it is advisable to fit the apparatus with a small inlet tap, whereby drops of ether can be allowed slowly to fall into the upper part of the condenser during the distillation. By this means the emulsion is broken down so that distillation proceeds without any interference. The process is very slow indeed, and it requires many hours to distil over 10–20 g. of the oil. The distillate, after separation from water, is a pale lemon yellow coloured oil possessing the characteristic terpene-like smell of the original material. It gives in undiminished intensity the colour reactions to which reference has been made, and on administration to rats proves as potent a source of vitamin A as the

equivalent amount of original oil or unsaponifiable matter. The yield of active distillate corresponds to from 0·3 to 0·4 % of the original oil. The distillation residue, after dehydration, is a dark brown, amber-like gum, corresponding in weight to about 0·05 % of the original oil. It gives no response to the colour reactions, and is inactive when tested on animals.

Distillation of the Cholesterol-free Fraction under reduced pressure.

During the course of this work a large number of fractional distillations of the cholesterol-free fraction of the unsaponifiable matter from cod-liver oil have been carried out. The earlier experiments indicated that to effect any sort of separation of the constituents by this means would be no simple matter, primarily because the range of temperatures over which the fractionation is conducted is short. Therefore it was necessary to distil a reasonably large volume of liquid, to employ a fractionating column of suitable design and length, and carefully to maintain control of the temperature difference between the boiling and distilling fluid. With these precautions, and a series of refractionations, it was hoped to separate the chief constituents in a state sufficiently pure to enable one to determine their nature. Unfortunately, the work throughout has been hampered by the great difficulty of obtaining sufficient material to enable a satisfactory fractionation to be performed. In the earlier investigations quantities of from 20 to 50 g. of the cholesterol-free oil were frequently distilled. These preliminary trials, unsatisfactory as they were, gave us some indication of the results we might expect on a large scale experiment. Working at pressures of from 2–4 mm. we usually collected the distillate in four fractions [Drummond and Coward, 1924].

I. A very small fraction of a clear colourless liquid distilling over the range 40–70° (4 mm.). II. A clear, slightly yellow oil collected between 180–220° (3 mm.). III. A clear, pale yellow oil 220–260° (3 mm.). IV. A heavy, golden yellow oil 260–300° (3 mm.). The residue remaining behind in the flask was a very heavy reddish brown oil showing a marked green fluorescence. On cooling, it solidified to an amber-like resin. Of the volatile fractions, we have ignored the first on the ground that it is almost certainly derived from the ether used in the extraction. Owing to the small amount that has been available we have not succeeded in identifying it. It usually possessed an iodine value of the order of 20, indicating a saturated compound contaminated with traces of the unsaturated compounds in the higher fraction. Its odour resembled that of the higher ketones. Attempts to prepare a crystalline derivative failed. It had no effect on growth. Indirect light on its nature was obtained during some experiments on the reduction of certain fractions from the unsaponifiable matter with hydrogen in the presence of catalytic nickel. In these experiments a liquid of relatively low boiling point distilled over from the hydrogenation vessel. Its smell closely resembled that of octyl alcohol, and as far as the small amount available permitted us to establish

its identity the belief that it was one of the octyl alcohols was confirmed[1]. Our earlier studies of the substances present in the other fractions II, III and IV, led us to believe that they were largely composed of unsaturated alcohols [Drummond and Coward, 1924; Drummond, 1924], but the amounts obtained were quite inadequate to enable us to determine their nature. A few details may be given of a typical distillation of this type. 69 g. of unsaponifiable matter—representing about 10 litres of a light coloured Lofoten oil—yielded a total of 33 g. of cholesterol and just over 30 g. of the orange coloured oil. This was separated from resins by distillation in a current of superheated steam and nitrogen, and yielded 25 g. of a clear, pale yellow oil. On distillation at 3 mm. pressure this oil yielded the following fractions:

(*a*)	100°	...	...	0·20 g.
(*b*)	100°–180°	...	...	1·2
(*c*)	180°–230°	...	...	8·29
(*d*)	230°–240°	...	...	1·40
(*e*)	240°–260°	...	...	6·23
(*f*)	260°–300°	...	...	1·73
Undistilled residue		...	...	3·5
				22·55 g.

It must be stated, however, that on many occasions the rate of distillation from 180° to 300° was steady. In the experiment described it was only possible to attempt redistillations of fractions (*c*) and (*e*), and these yielded respectively 5 g. of a clear lemon-yellow coloured oil boiling between 200° and 220°, and 3 g. of a darker yellow oil boiling at 250–260° at 2–3 mm.

Vitamin A Content of Fractions.

The fractions (*b*)–(*f*), as well as the undistilled residue, were tested for vitamin A by feeding tests. These indicated that vitamin A passed over mainly between 180–220°, at 2–3 mm. These experiments conclusively proved that the vitamin is volatile at these temperatures (3 mm.), and that very little loss of activity takes place during the operation[2]. It may be said, in passing, that throughout this research we have noted a perfect agreement between the intensity of the colours yielded by the fractions on applying the sulphuric acid test [Drummond and Watson, 1922] or the later substitutes for that test [Rosenheim and Drummond, 1925] and the activity of the fractions as determined by feeding experiments on rats.

[1] The occurrence of impurities, mainly mixed ketones, derived from the ether has been a frequent source of trouble to us. We first fully appreciated its disturbing effect when engaged in the fractionation of a large batch of unsaponifiable matter. During the extraction of this material we concentrated in our product the less volatile of the impurities of approximately 200 l. of ether. At later stages in the separation of the active fractions we obtained a total of nearly 50 cc. of mixed ether impurities. Fractionation of this material yielded four main fractions, all possessing the typical smell of the higher ketones. The presence of ethylmethyl ketone, B.P. 81°, diethyl ketone, B.P. 102°, and another ketone, B.P. 130°, was indicated by an examination of these fractions.

[2] Curiously, we have experienced rather serious losses of activity during the refractionation of the initial fractions, in spite of all attempts to conduct the later distillations with as great, or even greater, care. As yet we have no satisfactory explanation of this occurrence.

Preliminary Chemical Examination of Main Fractions (c) and (e).

Although it was obvious that the redistilled fractions (*c*) and (*e*) were probably impure, they were submitted to a superficial examination in order to ascertain something of their nature. The results of this examination indicated, as we had expected, that the chief constituents were unsaturated alcohols. Thus the averages for a number of determinations of the iodine value on various samples of each product were: (*c*) 110, (*e*) 130. The corresponding acetyl values were (*c*) 170, (*e*) 164, and the approximate molecular weights, (*c*) 320, (*e*) 350; neither fraction was optically active. With the sure knowledge that our products were impure, it was obviously unsafe to attach more importance to these data than merely regarding them as indications that unsaturated alcohols probably formed a considerable proportion. Certainly it would have been absolutely unjustifiable to regard either one of these preparations as the vitamin A in even approximately pure condition.

Detection of an Unsaturated Hydrocarbon in Cod-liver Oil.

In the preliminary study of these fractions it was ascertained that on treatment with an excess of bromine in anhydrous ether solution a slow deposition of a heavy white microcrystalline compound occurred. The manner in which this substance was precipitated and its general appearance suggested a similarity to the ether-insoluble dodecabromide of the curious unsaturated hydrocarbon that has been found in liver oils from other species of fish. This hydrocarbon has been termed squalene by Tsujimoto [1916, 1920] and given the formula $C_{30}H_{50}$, whereas Chapman [1917, 1918, 1923] believes it to be represented by $C_{29}H_{48}$ and calls it spinacene. The bromide from the cod-liver oil fractions is almost insoluble in dry ether (0·11 % at room temperature), and may be recrystallised in a rather unsatisfactory manner by dissolving in tetrachloroethane, in which it is fairly soluble, filtering and reprecipitating by the addition of alcohol or dry ether. There remains little doubt that the bromide prepared from the cod-liver oil fraction is identical with the bromide of spinacene $C_{30}H_{50}Br_{12}$. Estimations of bromine by Carius' method gave values of 68·57 %, as compared with 70·07 % required for the formula $C_{30}H_{50}Br_{12}$, or 70·80 % required for $C_{29}H_{48}Br_{12}$[1]. It shows no definite melting point, but on heating darkens at about 160°, and decomposes without sharp melting at 180–190°. The presence of spinacene in our fractions was confirmed by the preparation of the typical hexahydrochloride by passing dry hydrochloric acid through the solution of the substance in dry ether or dry ethyl alcohol until it is saturated. All our fractions yielded a precipitate of the characteristic crystalline hydrochloride, which can be readily obtained in a pure condition by recrystallisation from acetone. A large number of preparations from our fractions showed close resemblance to the hydrochloride prepared from specimens of pure spinacene kindly provided by Mr Chaston Chapman, F.R.S. Melting point varies from 110–125°, chlorine content 33·70,

[1] Owing to the difficulty of purifying the bromide we have failed to get figures for the bromine content close to the theoretical, even when analysing the product prepared from pure spinacene.

33·52 %. Calculated for $C_{30}H_{56}Cl_6$, 33·86 %; $C_{29}H_{54}Cl_6$; 34·63 %[1]. The detection of spinacene in all our fractions, and our inability to separate it from the other components by refractionations, impressed us with the difficulties which faced us in our efforts to determine the nature of the vitamin A. Indirect evidence that this hydrocarbon was not responsible for the effect on growth was provided by the fact that the fractions of highest boiling point tended to be richest in spinacene, but of relatively low vitamin value. Direct tests on a sample of unsaponifiable matter containing approximately 85 % of spinacene, for which we are indebted to Mr Chapman, as well as on a pure specimen made by us from the liver oil of *Spinax niger*, confirmed this view. Spinacene yields no response to the colour reactions we have referred to.

Separation of a Solid Saturated Alcohol (Batyl alcohol?).

It was observed that a small amount of a crystalline substance separated from the fractions of higher boiling point on standing for several days. On removing the crystals and recrystallising from acetone or methyl alcohol, a very small amount of a clear white product was obtained. There was just sufficient material to serve for a feeding test on two rats, which demonstrated its inactivity. The substance melted at 60°, was saturated, and was, we think, the same substance as is described below.

A solid substance closely resembling the product from cod-liver oil was isolated from the liver oil of *Spinax niger*. The unsaponifiable matter represents some 80 % of this oil and is almost entirely composed of spinacene. Purification of the spinacene by treatment with methyl alcohol, in which it is very slightly soluble at 0°, yields a fraction of more soluble material from which the crystalline substance separates in appreciable amounts. On recrystallisation from methyl alcohol we have obtained a preparation with a melting point as high as 65°. The Japanese investigators give the melting point of batyl alcohol as 69°. The crystalline form resembles that of the compound obtained from cod-liver oil[2].

Further manipulation of our fractions gave no satisfaction. We were quite unable owing to the very small amount of material available to isolate any other substances or their derivatives in the pure condition.

A consideration of our progress up to this point made us realise that only two paths were open by which further advances could be made. One would be to repeat our previous work on a much larger scale, hoping thereby to obtain fractions suitable for repeated refractionation, whereas the other would be to gather indirect evidence as to the nature of vitamin A by the examination of the unsaponifiable material in certain other fish-liver oils. Both these paths have been traversed during the last two years with, we fear, indifferent success.

[1] The melting point of the hydrochloride is uncertain. Repeated recrystallisation from hot solvents causes a diminution in chlorine content. The substance twice recrystallised from hot acetone usually melts about 115°.

[2] Four analyses of the solid alcohol gave figures close to the averages of C 72·29 %; H 12·48 %. Batyl alcohol, if it possesses the formula $C_{20}H_{40}O_3$ requires C 72·7 %; H 12·7 %.

Large Scale Fractionation of Unsaponifiable Material from Cod-liver Oil.

We originally intended to prepare material for this study by saponifying a large quantity of cod-liver oil on a small technical scale. Mr E. R. Bolton, of Technical Research Works, Ltd., undertook to prepare material in his experimental plant according to our directions. A certain amount of oil was treated in this manner, but, in spite of very considerable care, it was found that rather serious loss of vitamin activity had occurred during the process. As opportunity for investigating the cause of the inactivation did not immediately present itself, we fell back on a large-scale laboratory preparation in which 5–7 litres of oil were saponified at one time. We wish to express our indebtedness to Mr Bolton and his co-worker, Mr Lush, for the help and advice they so generously gave.

The collection of unsaponifiable material by the alternative method was somewhat tedious, and we suffered the disappointment of losing a considerable proportion of the total of more than a kilogram owing to an "ether peroxide" explosion in the final stage of preparation. Sufficient material was saved, however, to enable us to carry out a separation on a much larger scale than had previously been attempted. The general lines along which the separation was conducted have been already described. 750 g. of unsaponifiable matter yielded a total of 370 g. of cholesterol (49·3 %). It was during the concentration of the cholesterol-free fraction of this preparation that nearly 50 cc. of mixed impurities of ether were obtained (see footnote, p. 1054). The fractionation under reduced pressure of a portion of the cholesterol-free oil was carried out with great care, employing a fractionating column and adequate "lagging." The initial fractionation yielded just over a gram of a clear liquid boiling below 40° at 3–4 mm. This fraction was apparently identical with that described on p. 1053, which we believed to be a C_8 ketone, derived from the reagents employed in the extraction. The distillation of the main bulk of the oil failed to give us a well-marked separation, being fairly regular throughout after 150°.

The following fractions were collected and on examination gave the data:

Fraction	Description	Temperature Range	Quantity g.	Iodine value	Acetyl value	Colour reaction[1]
II	Very pale yellow oil, marked terpene-like odour	125°–155° (3 mm.)	5	114	.	− +
III	Pale yellow oil, terpene odour	155°–184° (2 mm.)	7	120	196	+ +
IV	Yellow oil, similar smell	184°–190° (2 mm.)	6	125	120	+ + + +
V	Yellow oil, pungent terpene-like smell	190°–220° (1–2 mm.)	36	129	174	+ + + +
VI	Orange yellow oil, pungent smell	220°–270° (1–2 mm.)	27·5	149	140	+ +
VII	Reddish brown amber-like resin	Residue 270°	20·5	.	.	−

[1] We invariably employed the colour reactions as a preliminary indication of the presence of vitamin A, reserving the animal tests for the examination of the more important fractions. On no single occasion in the course of five years' work has this procedure misled us.

Attempts were made to refractionate V and VI, but without any real success, and we were reluctantly forced at length to conclude that, without possessing very much larger quantities of material, there was little hope of separating the constituents satisfactorily by this means. A considerable amount of confirmation of our earlier results was, however, obtained by an examination of the redistilled fractions V and VI. Spinacene was present in both fractions, but to a considerably greater extent in that of higher boiling point, which also yielded an appreciable amount of the solid, saturated alcohol (M.P. about 60°) referred to previously. Sufficient of the substance was not obtained to purify it adequately, much less to establish its nature, but we have reasons for believing that it is identical with the substance described on p. 1056 as being isolated from the liver oils of the fish, *Spinax niger*. We were able to confirm its inactivity when tested on animals.

Attempted Fractionation by means of Solvents.

Having failed to separate the constituents of V and VI by distillation, we turned to fractional solution as a means of achieving our aim. These oils are extremely soluble in most fat solvents, ether, light petroleum, chloroform, carbon disulphide, carbon tetrachloride, tetrachloroethane, trichloroethylene, but are less soluble in methyl and ethyl alcohols and acetone. Takahashi and Kawakami [1923] reported that the cholesterol-free fraction of cod-liver oil unsaponifiable matter yields on recrystallisation from 80–90 % methyl alcohol at − 20° a semi-crystalline substance, which they regarded as vitamin A in the nearly pure condition[1]. We repeated their experiments, testing not only methyl and ethyl alcohol but also acetone as a solvent. Slow crystallisation at temperatures about − 10° throws down a flocculent, white precipitate, which, seen under the microscope, consists of bunches of slender needle-shaped crystals mixed with fine droplets of oily substances. The crystals melt about zero, and a pale yellow oil is produced. This product was examined chemically, as well as for vitamin activity. As we reported some time ago, it is certainly not vitamin A [Drummond and Coward, 1924] because it is less active in promoting growth than the mother liquors. Furthermore, the material thrown down from solution on cooling tended to be decidedly richer in spinacene than the original fraction, as indicated by the iodine values and the yields of insoluble bromide on treatment with bromine. The application of these processes of crystallisation did not seem to facilitate the purification of any of the constituents present in our fractions, and we abandoned them. We also attempted to effect some separation by means of differential solubilities in mixtures of solvents. Our exploration in this direction was very superficial, being limited to an examination of the so-called "phase test," frequently employed in the separation of the lipochrome pigments. 2 g. of an active fraction were dissolved in 20 cc. of light petroleum and shaken up vigorously at room temperature with an equal volume of methyl alcohol. On separation

[1] This claim to have isolated vitamin A is an earlier one than that criticised on p. 1062. The active substance was then believed to be an aldehyde.

of the liquids, of which the light petroleum was distinctly the more deeply coloured, and removal of the solvents, two fractions were obtained. The fraction soluble in methyl alcohol weighed 0·48 g. It possessed an iodine value of 116, gave a strong colour reaction, and produced growth in the test rats. The light petroleum fraction weighed 1·42 g., had an iodine value of 129, and was of definitely higher vitamin value.

At the time the results did not appear to be particularly encouraging, and the experiments were discontinued, but it is not unlikely that it would be worth while making more careful studies in this direction.

Nature of the Unsaturated Alcohols Present.

The results obtained seemed to indicate that apart from spinacene, the saturated alcohol (M.P. about 60°), and possibly unchanged lipochrome pigment, the bulk of the cholesterol-free fraction consisted of one or more unsaturated alcohols. In our earlier studies we were inclined to believe, from the progress of the fractional distillations, that two such alcohols were present, one boiling about 210° (3 mm.) and the other about 250° (3 mm.) [Drummond and Coward, 1924]. Later work, in which larger quantities of material were employed, has tended to weaken this view, and our inability to obtain any satisfactory fractionation leads us to believe now that probably only one unsaturated alcohol is present in any appreciable quantity. Our examinations of the fractions for this substance have been greatly impeded by the difficulty of removing the spinacene, or of forming a trustworthy opinion as to the amount of that hydrocarbon present. An attempt was made to estimate the amount of spinacene present by weighing the yields of dodecabromide and hydrochloride. Unfortunately, our experience leads us to confirm the view shared by Chapman and by Tsujimoto that both reactions proceed abnormally and that they cannot be regarded as quantitative. Of the two reactions, that of the formation of the hydrochloride is without doubt the more reliable. A series of estimations of the yield of this compound obtained from specimens of practically pure spinacene indicates that the yield of hydrochloride depends on the concentration of the hydrocarbon in the solution. In dry ether the yield varies from 30–50 %, whereas in dry ethyl alcohol higher yields, up to 60 % of the theoretical, have been obtained. The solubility of the hydrochloride is very slight in these solvents. Assuming that the precipitation of the hydrochloride is not interfered with by the other substances present—an admittedly unsound assumption—an attempt has been made to gain some idea of the proportion of spinacene present in the cod-liver oil fractions.

B.P. of fraction (3 mm.)	Iodine value	Weight taken g.	Solvent	Weight of hydrochloride	Yield assumed %	% spinacene in fraction
180°–200°	110	0·750	Dry ether	0·030	40	6·5
180°–200°	110	0·500	„	0·019	40	6·2
200°–220°	129	1·056	Dry alcohol	0·080	55	9·4
					Average	7·7

These figures, approximations as they are, may give us a rough idea of the amount of spinacene in our fractions, and are supported by those obtained when in a similar fashion attempts were made to determine the yield of dodecabromide. The yield of insoluble bromide appears to depend on a number of factors, such as the rate of addition of bromine, temperature, the time of standing, and in particular upon the presence of traces of moisture. Wide variations in yield, from 22–45 % of the theoretical, have been obtained using pure spinacene in dry ether. The yields of insoluble bromide given on bromination of the fractions from cod-liver oil were as follows:

B.P. of fraction (3 mm.)	Weight g.	Weight insoluble bromide g.
180°–200°	0·5100	0·0310
,,	0·5110	0·0495
,,	0·1010	0·0107

Assuming an average yield of 35 %, these amounts of bromide correspond to the following percentages of spinacene, 5·3, 8·4, 9·2, average – 7·6 %.

Whilst the unsatisfactory nature of these calculations must be admitted, we are inclined to believe that the approximate agreement between the figures given by the two methods indicates that the amount of spinacene present may be taken as 8 %. If we accept this figure as an approximate estimate of the percentage of spinacene in our physiologically active fraction, we may proceed to make one or two other calculations in the hope that they will throw some light on the nature of the other unsaturated constituents:

Iodine value. The iodine value of spinacene is 371. The average for a large number of active fractions is 124. This indicates an iodine value of about 103 for the spinacene-free residue.

Acetyl value. A number of determinations on active fractions gave values of 208, 173, 174, 210, 217, 202, 204, averaging approximately 198. Corrected for the presence of 8 % of spinacene this becomes approximately 215.

Molecular weight. Direct determinations by the cryoscopic method gave values of about 300.

These approximations suggest that the other main constituent or constituents of the active fractions might be unsaturated alcohols containing one hydroxyl group and one ethylene linkage (*e.g.* $C_{18}H_{35}OH$, M.W. 270, acetyl value 207, iodine value 94; $C_{20}H_{39}OH$, M.W. 296, acetyl value 189, iodine value 91·3).

Unsaturated Alcohols in Fish-liver Oils.

The work of Tsujimoto and his colleagues in Japan has provided much valuable information regarding the substances present in the unsaponifiable fractions from a variety of fish-liver oils. Tsujimoto and Toyama [1922] describe two alcohols in the liver oil of *Hexanchus corinus*. One of these is a solid substance to which they give the formula $C_{20}H_{42}O_3$, and the name batyl alcohol, whilst the second is an unsaturated alcohol, selachyl alcohol, $C_{20}H_{40}O_3$, which on reduction is converted into batyl alcohol.

Evidence has been obtained that the solid alcohol isolated by us from cod-liver oil and the liver oil of *Spinax niger* is probably the same compound as the Japanese investigators have described under the name of batyl alcohol. Selachyl alcohol appears to form a large proportion of the unsaponifiable matter derived from the liver oils of certain fish, *e.g. Cirrhigaleus barbifer*, *Somniosus microcephalus*, *Chimaera* (sp.), *Lepidorhinus kinbei* and *Zameus* (*Scymnodon*) *squamulosus*.

Another unsaturated alcohol, believed to be oleyl alcohol, $C_{18}H_{36}O$, has been found by Toyama [1922] as the main constituent of the unsaponifiable matter from the liver oil of *Chamylodoselachus anguineus*. In view of these observations it was necessary to determine whether these alcohols occur in cod-liver oil, and if so, whether they are related to the physiological activity.

Before presenting the evidence we have accumulated on these questions it will be well to refer to another path along which enquiries were made. Surveying the other unsaturated alcohols of high molecular weight that have been isolated from natural sources it was obvious that attention should be paid to the alcohol phytol, $C_{20}H_{39}OH$, derived from chlorophyll. It appeared quite likely that small amounts of phytol might be absorbed by the animal organism during the digestion of green plants, and that the alcohol might appear as a constituent of the unsaponifiable fraction of their liver fats. The nature of the unsaturated alcohols present in our active fractions is also bound up with the nature of the product described recently by Takahashi, Nakamiya, Kawakami and Kitasato [1925] under the name "biosterin," and regarded by them as pure vitamin A. The most complete account of their work is given in a monograph, printed in English, but there have been several earlier papers in Japanese [Takahashi, 1922; Takahashi and Kawakami, 1923].

The following table gives the data regarding the alcohols that have been mentioned, together with the corresponding data for spinacene and "biosterin."

Table I[1].

	Selachyl alcohol	Oleyl alcohol	Phytol	"Biosterin"	Our active fractions allowing for 8 % spinacene	Spinacene
% C	73·0	80·8	80·0	81·0	77·7	87·8
% H	12·2	13·5	13·5	11·0	11·8	12·2
Mol. weight	328	270	296	402	300	410
Formula	$C_{20}H_{40}O_3$	$C_{18}H_{36}O$	$C_{20}H_{40}O$	$C_{27}H_{46}O_2$	$[C_{20}H_{36}O_2]$	$C_{30}H_{50}$
Iodine value	79·15	94	91·3	180	124	371
Acetyl „	272 (dihydric)	207	189	139	215	—
Refractive index	N_{20} 1·4691	N_{20}^{D} 1·4620	N_{20}^{D} 1·4640	N_{20}^{D} 1·52517	N_{20}^{D} 1·4705	N_{20}^{D} 1·4965
Boiling point	236°–239° (5 mm.)	207° (13 mm.)	202° (10 mm.) 145° (0·02 mm.)	147° (0·02 mm.)	180°–220° (1–2 mm.)	252° (5 mm.) 240° (2 mm.) 203° (0·15 mm.)
Density	d_{20} 0·918	d_4° 0·862	d° 0·852 d° 0·864	d_{15}° 0·9561	—	d_{15} 0·8587

[1] The majority of the recorded values for oleyl alcohol, phytol, and spinacene have been checked during the course of this work on preparations made in our laboratory.

A superficial examination of the monograph published recently by the Japanese investigators might reasonably give the impression that they had isolated vitamin A in the pure state, but such a view is rapidly dispelled when the evidence they have presented is carefully examined, particularly in the light of a knowledge of the peculiar difficulties that are encountered in working with complex mixtures of fatty substances of high molecular weight, such as are represented by the unsaponifiable fractions of oils and fats.

Boiling point. The constancy of the boiling point of "biosterin" would at first sight appear to indicate its purity, but it has been our experience that although fractions boiling over narrow ranges can be obtained in distillations of such substances, these fractions are nevertheless often mixtures.

Thus, very active fractions have often been collected between 180–200° at 2–3 mm.—approximately the range at which "biosterin" would probably boil at the same pressure—but they have been found on examination to be highly impure. The difficulty of separating the components of these mixtures of substances boiling at temperatures close together is so generally recognised that it is difficult to understand how Takahashi and his colleagues can have imagined that they were obtaining a pure product after a single distillation from a modified form of retort. For our own part we recognise our failure to effect any appreciable separation of the constituents of the cholesterol-free material by repeated fractionation from Claisen flasks fitted with various types of fractionation columns, and suitably "lagged" to maintain control of the temperature difference between boiling and distilling liquid.

The difficulties encountered in these distillations were of the same type that we had previously encountered in an attempt to separate the esters of the fatty acids of butter by repeated fractionation [Channon, Drummond and Golding, 1924].

The description of "biosterin" as "a reddish yellow, transparent viscous, oily substance" is in our opinion clear evidence that the product must have been impure. Fractions obtained by us were usually coloured a pale lemon yellow unless the distillation had been unsatisfactory and bumping and splashing had occurred. The redistilled fractions were always crystal-clear, pale yellow oils, and only the material passing over at the higher temperatures was at all viscous. Incidentally, the alcohol phytol boils at the temperature given for "biosterin"—145–147° at 0·02–0·03 mm.—so that, whereas there may be little or no phytol in the unsaponifiable matter from cod-liver oil, there certainly must be considerable amounts present in a "biosterin" prepared from green leaves.

Analysis and molecular weight. "Biosterin" contains 81·0 % of carbon and 11·0 % of hydrogen, and is stated to have a molecular weight of about 400. It must be apparent that elementary analyses made on heavy oily materials of doubtful purity have very little value. Analyses of our own active fractions were made in order to gain information as to the amount of oxygen-containing substances (alcohols) present, but it would be unreasonable

to attach any importance to the fact that many of these analyses can be made to fit formulae, *e.g.* C 77·7 %, H 11·8 %, and cryoscopic determinations giving values about 300 correspond very well with the formula $C_{20}H_{36}O_2$.

Unsaturation. "Biosterin" is reported to contain three double bonds corresponding to an iodine value of 190. The highest iodine value recorded for any of our active fractions is 140, and it is known that this fraction contained at least 8 % of spinacene which has an iodine value of 371.

The "biosterin" prepared from cod-liver oil was found to contain 80·87 % C and 11·13 % H, whilst that prepared from green leaves contained 79·88 % C and 11·88 % H. These products were of similar physiological activity although their iodine values were 180 and 125 respectively. This large difference in iodine values of products of similar physiological activity would seem significant. The description of the preparation of "biosterin" from plant tissues leaves no doubt that considerable amounts of phytol (iodine value 91) must have been present.

The yield of the bromide of "biosterin," prepared by the addition of bromine dissolved in glacial acetic acid to a solution of "biosterin" in ether, is stated to have been 85 %. This we are quite unable to confirm, for, as stated elsewhere, bromination of our fractions yielded only small amounts of the characteristic insoluble bromide of spinacene.

Refractive index and specific gravity. The refractive index of "biosterin" is given as 1·52517 and the specific gravity at 15° as 0·9561. Both values are much higher than any we have encountered during our work, or the values given by components such as spinacene, and we are inclined to regard these figures with suspicion.

Physiological activity. If we take as a measure of the physiological activity of the products the dose required to maintain good growth in a rat of 90–100 g. weight, it appears that the original cod-liver oil from which Takahashi obtained "biosterin" was represented by 1×10^{-3} g., whilst that for the majority of oils used by us was $5\text{–}6 \times 10^{-3}$ g. Our active fractions gave growth in daily doses of 5×10^{-5} g. whilst "biosterin" did so in a dosage of 1×10^{-5}–5×10^{-6} g. In other words, the degree to which the vitamin has been concentrated in "biosterin" and our active fractions is of the same order if regard be paid to the activity of the original oils, and yet we know that our preparations were highly impure. We are entirely at a loss to account for the observation of the Japanese investigators that harmful results follow the administration of an excess of the active substance. We have many times administered large amounts of highly active fractions without observing any ill effects, and the suspicion is aroused that Takahashi's material may have contained traces of a toxic impurity.

Action of "biosterin" on sensitised plates. The description of the experiments made by Takahashi and his colleagues on the influence of their active material on photographic plates leaves no doubt that they were observing the effect described by Russell [1908]. No "fogging" occurred when quartz or glass

separated the plate from the "biosterin." These observations provide additional confirmation that materials possessing vitamin A activity do not emit a radiation capable of "fogging" a sensitised plate after passage through quartz, as was originally claimed by Kugelmass and McQuarrie [1924]. It was shown by Drummond and Webster [1925] that the experiments of the latter were faulty, and that the "fogging" was probably due either to the "Russell effect" or to the action of a phosphoresence which is emitted by silica after it has been exposed to ultra-violet light. Kugelmass and McQuarrie have recently admitted their mistakes [1925].

Attempt to determine the Nature of the Unsaturated Alcohols present in the Active Fraction from Cod-liver oil.

The iodine value and acetyl value, after correction for spinacene, suggest that the unsaturated alcohols contain one ethylene bond and one hydroxyl group capable of acetylation. The possibility of oleyl alcohol or phytol being present had to be investigated.

Both these alcohols yield fairly characteristic crystalline silver salts after conversion into the acid phthalic esters. Treatment of our active fractions with phthalic anhydride in boiling benzene solution appeared to bring about little or no combination, and only a few crystals of what was possibly a silver salt were obtained. The amount was too small for examination. Control experiments with samples of oleyl alcohol prepared from oleic ethyl ester by the method of Bouveault and Blanc [1904], and with phytol prepared from phaeophytin [Willstätter and Stoll, 1913] gave good yields (60–70 %) of the characteristic silver salts. It was also ascertained that the presence of spinacene does not interfere with the formation of these compounds. Takahashi has also recorded that his active fraction does not yield an acid phthalate by the usual method, but that combination occurs at the higher temperature of 120–130°. The description of his phthalate is, however, somewhat unsatisfactory. From this evidence and from a failure to obtain the phenylurethanes we concluded that neither phytol nor oleyl alcohol is present to any marked extent in our active fractions. Incidentally, it is of interest to mention that Javillier, Baude and Lévy-Lajeunesse [1925] showed by feeding experiments on rats that phytol does not possess the physiological action of vitamin A. Our preparations of phytol and oleyl alcohol failed to give a response to the colour tests for vitamin A, and feeding experiments also gave negative results. There remains the possibility that the selachyl alcohol described by Tsujimoto might be present, and that it might be responsible for vitamin action. As far as we are aware no derivative of selachyl alcohol has been described that is suitable for its detection, so that no means are available for ascertaining the first point. Indirect evidence that this substance is not vitamin A is given by the fact that the liver oil of *Scymnodon squamulosus*, of which the unsaponifiable matter consists almost entirely of spinacene and selachyl alcohol, has been found by us to be physiologically of very low activity.

The evidence presented above seems to rule out the possibility of vitamin A being identified with oleyl alcohol, phytol or selachyl alcohol. In our opinion the data available are quite inadequate to tell us whether or not the physiological activity is due to the presence of other unsaturated alcohols, for no separation of these in a state of purity has been achieved. No satisfactory answer to this question can be given until the active constituent has been isolated in pure condition or as a derivative suitable for purification. It was of interest to observe that during the course of exhaustive experiments to obtain solid derivatives suitable for purification and examination the vitamin activity of our fractions survived certain chemical treatments of the hydroxyl group of the alcohols present, *e.g.* acetylation, benzoylation and treatment with phthalic anhydride or substituted benzoyl chlorides, but in no case did it survive exposure to reagents that affected the unsaturated linkages, *e.g.* bromination, reduction.

Other Constituents of the Unsaponifiable Fraction.

It is obvious that the unsaponifiable fractions from oils such as cod-liver oil are highly complex mixtures which have as yet been but imperfectly examined. Amongst the components are the lipochrome pigments first detected by Salkowski [1863]. The yellow pigments of cod-liver oil remain unidentified, and neither the fresh oils nor the concentrated fractions present absorption spectra which it has been possible to measure. It has been found during the course of this work that there is a rough correlation between the physiological activity of the oils and the degree of pigmentation but the significance of this is uncertain. It is also uncertain whether the pigments are volatile during distillation.

The association of vitamin A with the lipochrome pigments has been observed many times [Steenbock, 1919; Steenbock and Boutwell, 1920; Rosenheim and Drummond, 1920], but a number of exceptions having been described [Palmer and Kempster, 1919, 1, 2, 3; Drummond and Coward, 1920], it has been generally concluded that it is of no significance as regards the identity of the former substance. Drummond [1919] observed that a pure specimen of recrystallised carotene possessed no growth-promoting powers, whereas Steenbock has once reported that a specimen of carotene induced growth [Steenbock *et al.*, 1921]. No confirmation of this statement has appeared from their laboratory as far as we are aware.

More recent studies that we have made on a specimen of carotene recrystallised four times, M.P. 167·5°, suggest that the lipochrome possesses no growth-promoting activity such as vitamin A shows, but that it may, unless carefully purified, be contaminated by traces of the latter substance. This, at least, appears to be the most satisfactory explanation of the long survival of the animals in some tests when compared with the rapid failure of those on the basal ration. Lycopin, prepared from tomatoes, is likewise inactive. We are convinced, therefore, that neither carotene nor lycopin is vitamin A.

Xanthophyll has not yet been prepared by us in sufficiently pure condition for a satisfactory test.

SUMMARY.

1. Further confirmation has been obtained of the concentration without loss of vitamin A in the unsaponifiable fraction of cod-liver oil, provided the preparation is carried through with precautions against oxidation.

2. The concentrate contains no detectable traces of iodine or nitrogen, so that these elements do not appear to be related to the physiological action of the oil in promoting growth.

3. Approximately 50 % of the unsaponifiable matter from cod-liver oil is cholesterol, which may be removed quantitatively without loss of vitamin activity.

4. Vitamin A is volatile in superheated steam.

5. Distillation of the cholesterol-free residue in a high vacuum did not result in a satisfactory separation of the components. Vitamin A passes mainly between 180–220° at 2–3 mm.

6. Chemical examination of active distillates indicated the presence of (*a*) a saturated solid alcohol (M.P. ca. 60°), (*b*) the unsaturated hydrocarbon spinacene, (*c*) one or more than one unsaturated alcohol, boiling about 200° at 2–3 mm.

7. Spinacene and the solid alcohol are without vitamin A action.

8. It has not been possible to decide whether vitamin A is identifiable with one of the unsaturated alcohols.

9. The unsaturated alcohols, oleyl alcohol ($C_{18}H_{36}O$) and selachyl alcohol ($C_{20}H_{40}O_3$), which have been described as constituents of other liver oils, are not identifiable with vitamin A. The unsaturated alcohol, phytol ($C_{20}H_{40}O$), which is present in the chlorophyll molecule, is also devoid of activity.

10. A criticism is made of the claims of Takahashi to have isolated vitamin A and to have identified it as an unsaturated alcohol $C_{27}H_{46}O_2$ ("biosterin").

11. Brief reference is made to the possible relation between the lipochrome pigment of cod-liver oil and the vitamin activity of the latter.

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REFERENCES.

Bennett (1841). Treatise on Oleum Jecoris Aselli, London.
Bouveault and Blanc (1904). *Bull. Soc. Chim.* **31**, 1206.
Chapman (1917, 1918, 1923). *J. Chem. Soc.* **111**, 56, **113**, 458, **123**, 769.
Channon, Drummond and Golding (1924). *Analyst*, **49**, 311.
Drummond (1919). *Biochem. J.* **13**, 81.
—— (1924). *J. Soc. Chem. Ind.* **43**, 928.

Drummond and Coward (1920). *Biochem. J.* **14**, 668.
—— —— (1921). *Lancet*, ii, 698.
—— —— (1922). *J. Soc. Chem. Ind.* **41**, 561 R.
—— —— (1924). *J. Soc. Chem. Ind.* **43**, 544.
Drummond and Watson (1922). *Analyst*, **47**, 341.
Drummond and Webster (1925). *Nature*, **115**, 837
Funk (1914). Die Vitamine (Wiesbaden).
—— (1915). *Biochem. Bull.* **4**, 365.
—— (1916). *Amer. Pat.* **1**, 162, 901.
Gmelin (1837). *Ann. Pharm.* **29**.
—— (1838). *Ann. Pharm.* **31**, 323.
Hupfer de l'Orme (1836). *Hufeland's J.* **8**, 115.
Javillier, Baude and Lévy-Lajeunesse (1925). *Bull. Soc. Chim. Biol.* **7**, 39.
Kugelmass and McQuarrie (1924). *Science*, **60**, 272.
—— —— (1925). *Science*, **62**, 87.
McCollum and Davis (1914). *J. Biol. Chem.* **19**, 245.
Palmer and Kempster (1919, 1, 2, 3). *J. Biol. Chem.* **39**, 299, 313, 331.
Potemka, Martius and Sarphati (1838). *Med. Z. Heilk. Preussen*, **33**, 15.
Rosenheim and Drummond (1920). *Lancet*, i, 862.
—— —— (1925). *Biochem. J.* **19**, 753.
Russell (1908). *Proc. Roy. Soc. Lond.* B. **80**, 376.
Salkowski (1886). *Z. anal. Chem.* **26**, 565.
Springmüll (1837). *Pharm. Centr.* 1814.
Steenbock (1919). *Science*, **50**, 352.
Steenbock and Boutwell (1920). *J. Biol. Chem.* **42**, 121.
Steenbock, Sell, Nelson and Buell (1921). *J. Biol. Chem.* **46**, xxxii.
Takahashi (1922). *J. Chem. Soc. Japan*, **43**, 826.
Takahashi and Kawakami (1923). *J. Chem. Soc. Japan*, **44**, 590.
Takahashi, Nakamiya, Kawakami and Kitasato (1925). Publication of Inst. Phys. and Chem. Research, **3**, 81.
Toyama (1922). *Chem. Umschau.* **29**, 237, 245.
Tsujimoto (1916). *J. Ind. Eng. Chem.* **8**, 889.
—— (1920). *J. Ind. Eng. Chem.* **12**, 600.
Tsujimoto and Toyama (1922). *Chem. Umschau.* **29**, 27, 35, 43.
Williams (1912). *Brit. Med. J.* ii, 700.
—— (1913). *Pharm. J.* **89**, 806.
Willstätter and Stoll (1913). Untersuchungen über Chlorophyll (Berlin).
Zilva and Drummond (1923). *J. Soc. Chem. Ind.* **42**, 185 T, 250 T.
Zucker (1922). *Proc. Soc. Exp. Biol. Med.* **20**, 136.
Zucker, Pappenheimer and Barnett (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 167.