Studies in Vitamin A

12. WHALE-LIVER OIL ANALYSIS: PREPARATION OF KITOL ESTERS

By R. K. BARUA AND R. A. MORTON

Department of Biochemistry, University of Liverpool

(Received 9 March 1949)

Whale-liver oil is valued for its vitamin A content, but this has so far been difficult to determine. Indeed, there has been no satisfactory alternative to the biological assay, which is itself of limited utility, if only because the fiducial limits of error ($P=0.95$), using eighty rats, may often be no better than 70–140% of the estimated potency. Whale-liver oil is, moreover, a very variable material. The crude oil, obtained by processing livers at sea or in Antarctic stations, usually contains phospholipins, water, free fatty acids and vitamin A decomposition products, as well as other impurities. In addition there is kitol, a substance not known to occur in comparable quantity in any other fat. The refined whale-liver oils of commerce also vary considerably. Quite apart from the fairly wide range of vitamin A content in crude oils, the refined oils are obtained in different ways and the resulting products are better described as concentrates. In some, the vitamin A may exist mainly in ester form, and in others it may be predominantly present as the free alcohol. The concentrates are often relatively free from oxidation products derived from vitamin A, but they are rarely, if ever, free from kitol. Anhydrovitamin A (almost certainly an artifact) is also present to a variable extent in whale-liver oil samples.

Some decomposition of vitamin is perhaps unavoidable as a result of the difficulties inherent in working up in factory ships large amounts of liver containing only a low percentage of fat. Opinions have differed as to whether kitol is or is not present in the liver of the living animal. In any case the presence of anhydrovitamin A, of oxidation products and of kitol, gives rise to serious difficulties when the ultraviolet absorption or the antimony trichloride colour test is used to determine vitamin A in whale-liver oils or concentrates. Among the interfering substances, kitol is specially interesting because its biogenesis and possible function await elucidation.

Edisbury, Morgan & Morton (1935) found that, compared with fish-liver oils, whale-liver oils and concentrates exhibited anomalous ultraviolet absorption and abnormal colour tests, and Edisbury, Morton, Pritchard & Wilkinson (1937) described a fractionation of unsaponifiable extracts by partitioning between light petroleum and 83% (v/v)

aqueous ethanol. The fraction soluble in 83% ethanol contained nearly all the vitamin A, whilst the light petroleum-soluble fraction showed an ultraviolet absorption curve with a maximum at 285–290 m μ . and gave a purple rather than a blue colour with the antimony trichloride reagent ($\lambda_{\max}$ 594 and 496 m μ). The $E_{1\text{ cm}}^{1\%}$ (285 m μ .) value was raised to 240 (which would to-day be regarded as indicating 30–35% kitol), and the biological assay indicated about 17,900 i.u./g., corresponding possibly to about 0.5% of residual vitamin A or a very low intrinsic potency for the new substance.

Embree & Shantz (1943) subjected to molecular distillation a de-sterolized whale-liver oil concentrate dissolved in a maize-oil fraction and eliminated vitamin A at 150°. The residue yielded a non-saponifiable fraction, which, after chromatography on alumina, yielded a resin exhibiting $\lambda_{\max}$ 290 m μ ., $E_{1\text{ cm}}^{1\%}$ 580, and in the colour test showing maxima at 428, 505 and 580 m μ . They named the main constituent kitol. When the material was again subjected to molecular distillation elimination began at 175° and became maximal at 225°. The product was, however, less pure than before, because the kitol had decomposed, yielding a certain amount of vitamin A.

Embree & Shantz (1943) reported the following composition and characteristic properties of kitol: Mol. wt. 575. Formula, $\text{C}_{40}\text{H}_{58}(\text{OH})_2$. Yield of vitamin A by thermal decomposition: 1 mol. vitamin/mol. kitol. $[\alpha]_{5461}^{25} - 1.35$. $\lambda_{\max}$ 286 m μ ., $E_{1\text{ cm}}^{1\%}$ 580.

Baxter, Clough, Kascher & Robeson (1947) obtained a similar product with $E_{1\text{ cm}}^{1\%}$ (290 m μ .) 586, and from it obtained kitol crystals (m.p. 88–90°) after first obtaining a crystalline di-*p*-phenylazo-benzoate. The pure kitol gave $E_{1\text{ cm}}^{1\%}$ (290 m μ .) 707 and the formula $\text{C}_{40}\text{H}_{58}(\text{OH})_2$ was confirmed. Geometric isomerism complicated the work on kitol esters. It is thus clear that kitol is a di(vitamin A), but its structure has not been elucidated and it cannot be 'depolymerized' by any method short of pyrogenic decomposition.

The present paper is concerned with (a) gaining further information about kitol, in particular whether it is or is not an artifact, and (b) the solution of the problem of analysing whale-liver oils for

vitamin A. The background of (b) is discussed later (p. 314).

Hickman (1943) has suggested that kitol is a detoxication product in the sense that an 'excessive' accumulation of vitamin A is prevented when the inactive dimeride is formed in the whale liver. This is a legitimate enough speculation, but even its narrower implications have yet to be studied. Thus, the kitol/vitamin A ratio might perhaps vary with the potency of the oil and perhaps with the age of the whale. On the other hand, if kitol were found to be a normal constituent of whale blood and milk the suggestion would need to be reconsidered.

EXPERIMENTAL

Whale milk Kitol

Only one sample of milk, and that from a fin whale, has reached us. It contained 16% fat (0.7% of unsaponifiable matter), approx. 380 i.u. vitamin A/100 g. milk and showed ultraviolet absorption consistent with the presence of some kitol (Fig. 1). Examination for kitol of milks from sperm whales and blue whales is desirable, but material is not easily obtained.

Fig. 1. Ultraviolet absorption spectrum of fin-whale milk lipid, 1.75% in cyclohexane, 1 cm.

Whale-liver oil

The crude commercial oil is usually very dark and highly viscous. Our early attempts to prepare pure kitol esters were hindered by the presence of free fatty acids and phospholipins in the material subjected to chromatographic adsorption. Much better results were obtained with acetone-soluble fractions, particularly those obtained in the laboratory from preserved liver. The ease with which phospholipins dissolve in light petroleum is a disadvantage, because they tend to accompany kitol esters in chromatography. The following section illustrates the difficulty.

Phospholipins of whale-liver oil. Crude sperm-whale-liver oil (2.212 g.) was extracted many times with acetone, and the insoluble residue (0.766 g.) was dried in a current of CO₂. The residue, dissolved in light petroleum, was poured on a column (30 × 1 cm.) of full-strength alumina. The chromatogram was developed with light petroleum. The eluate con-

tained 0.41 g. of material giving no colour with SbCl₃ reagent and only weak general absorption in the ultraviolet with a faint inflexion at 275 mμ.

Further development with light petroleum-ether mixture (4:1) gave 0.097 g. of a pale yellow oil, λ_{max.} 290 mμ., E_{1 cm.}^{1%} 121, and ether-ethanol mixtures gave 0.153 g. of pale oil, λ_{max.} 295 mμ., E_{1 cm.}^{1%} 157. Ether extracted most of the residual material. There seems to be in whale-liver oil a substance of which kitol is a component and possessing an E_{1 cm.}^{1%} value near 160.

By dissolving crude sperm-whale-liver oil in light petroleum and adding acetone, a phospholipin fraction is thrown out of solution. The insoluble material may be redissolved in petroleum and reprecipitated by means of acetone. If this is repeated several times the material adsorbing selectively at 290 mμ. can be removed. The residual phospholipin shows a feeble inflexion at 275 mμ., and after chromatography the various fractions exhibit E_{1 cm.}^{1%} (275 mμ.) 23.6–32.4. The petroleum-soluble phospholipin (0.61 g.) yielded 0.063 g. of unsaponifiable matter with λ_{max.} 290 mμ., E_{1 cm.}^{1%} 40. Most of the ultraviolet absorption of the phospholipin was thus due to saponifiable constituents, but a small amount of material which does not give a colour with the SbCl₃ reagent is present and remains to be accounted for.

Although the main point of this section is to show the need for eliminating phospholipins it is clear that they are likely to repay further study. It is possible that kitol may play a part in glyceride-phospholipin exchanges. It may well be that an investigation of the lipids of the absorptive portions of the whale intestinal tract would be worth while, in spite of the formidable difficulties of collecting and preserving the material.

Frozen whale liver as a source of oil

Large portions (50 kg.) of fresh livers of sperm whale (*Physeter catodon*, syn. *Physeter macrocephalus*) and blue whale (*Balaenoptera musculus*) were frozen right through in the Antarctic. The liver was brought to England in the ship's refrigerator and thereafter kept in a commercial cold store, and portions were chopped off as required.

Blue-whale liver. A portion (approx. 2 kg.) of liver was thawed, sliced and minced. The mince was extracted several times with cold acetone. The combined, concentrated, acetone extract was mixed with water and the fat extracted with light petroleum. The combined extracts were washed with water, dried over Na₂SO₄ and the solvent was removed. The product was 40 g. of yellow oil which became brown on standing; sp.gr. about 0.95; I₂ val. 163.4; saponification equiv. 330.8. The ultraviolet absorption curve showed λ_{max.} 328 mμ., E_{1 cm.}^{1%} 36.7 (gross), 29.8 after correction according to the procedure of Morton & Stubbs (1946). The curve (Fig. 2) was not very abnormal compared with that of a pure vitamin A ester. The kitol content was not high.

The liver debris was ground and re-extracted with ether-ethanol mixture (3:2 v/v). The filtered extract was diluted with water and re-extracted with ether. The solute (2 g.) contained very little vitamin A or kitol, but some decomposition products were present.

The oil from this sample of frozen blue-whale liver contained much less kitol than vitamin A, and there was relatively little interference with spectrophotometric determinations of vitamin A.

Sperm-whale liver. The oil obtained by extraction of minced liver with cold acetone was light brown in colour but darkened on standing; I_2 val. 149.8, saponification equiv. 378.5. The ultraviolet absorption curve showed a very flat maximum near $310\text{ m}\mu$, $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$), 193.5; (286 $\text{m}\mu$) 122.9 (gross) (Fig. 3). The oil contained larger absolute amounts of vitamin A and kitol than that from blue-whale liver, and indeed a rough approximation indicated some 16% of vitamin A esters and perhaps 20% of kitol esters. The liver debris again gave a small yield of residual ether-soluble material.

Fig. 2. Ultraviolet absorption spectrum of cold acetone extract of blue-whale liver $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 36.7 (gross), 29.8 (corrected); solvent, cyclohexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

Fig. 3. Ultraviolet absorption spectrum of cold acetone extract of sperm-whale liver. $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 193.5 (gross), 141.5 (corrected); solvent, cyclohexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

The oil from this sample of sperm-whale liver was thus a good source both of vitamin A and kitol, but, as the latter was present in large amount, con-

siderable interference occurred in the spectrophotometric determinations for vitamin A.

The experiments on well-preserved frozen livers suggest that in commercial whale-liver oils the presence of vitamin A decomposition products is a more serious analytical obstacle than the presence of kitol. This point is reinforced by the results of tests on salted minced whale livers. It is not necessary to go into all the experimental details, but it may be said that the oil samples obtained by various methods of extraction were fairly uniform, the $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) values (uncorrected) varying from 37 to 60. The percentage of unsaponifiable matter was about 25. Anticipating the discussion of the analytical problem (see p. 314), the fraction of the gross absorption at 328 $\text{m}\mu$. due to vitamin A is about two-thirds and a conversion factor of 1200 is roughly correct. Attention is drawn at this stage to the fact that the oils obtained in the laboratory from preserved whale livers show the type of absorption spectrum to be expected from the summation of contributions due to kitol and vitamin A. From the point of view of analysis for vitamin A, the interference due to kitol is serious, but in commercial oils there is the additional difficulty of substantial irrelevant absorption due to artifacts.

Fractionation of whale-liver oils

Sand-column technique. Chevallier, Manuel & Faubert (1941) dispersed fish-liver oil on sand prior to extraction with solvents. The following experiment was carried out on the lines of their work: whale-liver oil concentrate (0.05 g.) was dissolved in 10 ml. redistilled ethyl ether, and the solution poured on to a column of fine, dry, silver sand ($2 \times 20\text{ cm.}$). After allowing the solution to seep into the sand the ether was removed by a brisk upward current of N_2 . The column was then washed with successive portions of solvent, with the results shown in Table 1. It was clear that 80% ethanol, if used in sufficient quantity, could extract anhydrovitamin A and vitamin A, leaving kitol behind.

Table 1. Elution of whale-liver oil concentrate dispersed on sand

Eluting solvent. Ethanol %	Volume (ml.)	$\lambda_{\text{max.}}$ ($\text{m}\mu$.)	$E_{1\text{ cm.}}^{1\%}$ *	Vitamin A $E_{1\text{ cm.}}^{1\%}$ * 328 $\text{m}\mu$.	
				Gross	Corrected
(a) 0.05 g. oil used					
70	270	328	—	42.4	33.6
80	540	328	—	50.8	26.6
90	90	292	17.6	—	—
100	90	290	25.3	—	—
(b) 0.05 g. oil used					
60	90†	328	—	19.04	7.84
70	90†	328	—	9.65	6.44
80	90†	328	—	19.04	15.96
90	90	328	—	38.8	30.1
100	90	295	53.2	—	—

* Calculated on weight of original oil.

† Solvent passed through column five times.

A further experiment was tried on a larger scale and sand was not used: 0.5105 g. of 'oil' was boiled with 80% ethanol, the solution cooled and the supernatant liquor decanted. The 80% ethanol-insoluble fraction (0.3075 g.) showed $\lambda_{\max}$ 310–313 m μ . and contained about 4% vitamin A and 14% kitol. The soluble fraction (0.199 g.) showed $\lambda_{\max}$ 328 m μ . and contained some 3% of vitamin A contaminated by anhydrovitamin A and free kitol. The 'insoluble' fraction was repeatedly washed with hot 80% ethanol, cooled and the supernatant liquid decanted. The residue showed $\lambda_{\max}$ 295 m μ ., $E_{1\text{ cm.}}^{1\%}$ 125, corresponding with some 18% of kitol or about 36% kitol esters. This fraction (0.16 g.) was boiled with considerable excess of ethanolic KOH and 0.1044 g. unsaponified residue was obtained containing about 4% vitamin A and 23% 'free' kitol. This material was chromatographed on full-strength alumina (Spence, Grade 0) using light petroleum as the developing solvent. The various fractions were examined spectrophotometrically (Table 2). If $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) = 700 for pure kitol the material from the top zone contained 84% kitol (cf. Fig. 4).

Table 2. *Chromatography of 0.08 g. of unsaponifiable material from the fraction of whale-liver oil concentrate insoluble in 80% ethanol*

Zones	Wt. obtained (g.)	$\lambda_{\max}$ (m μ .)	$E_{1\text{ cm.}}^{1\%}$ (286 m μ .)	$E_{1\text{ cm.}}^{1\%}$ (286 m μ .) with respect to unsaponifiable material
Top	0.0105	286	588	77.20
Second	0.0096	286	317.1	39.63
Third	0.0564	325	21.3	14.90
Fourth	0.0019	—	—	0.75
—	0.0784	—	—	132.48

The foregoing experiments suffer from the disadvantage of being on a very small scale. In an attempt to overcome this, 50 g. of oil were heated with absolute ethanol (1 l.) until the solution became clear, and water (250 ml.) was then added and the solution cooled. The mixture was then put through a Sharples centrifuge. The soluble portion showed $\lambda_{\max}$ 326.5 m μ . and contained most of the vitamin A and anhydrovitamin A. The insoluble portion was repeatedly extracted with small amounts of ethanol. The residue was chromatographed in light-petroleum solution on full strength alumina. After several chromatographic separations it became clear that under these conditions fractions showing $\lambda_{\max}$ 286 m μ ., $E_{1\text{ cm.}}^{1\%}$ 166 were the best obtainable.

Chromatography of whole oil on a small scale gave two main kitol fractions, $\lambda_{\max}$ 286–290 m μ ., $E_{1\text{ cm.}}^{1\%}$ about 130 and 315, respectively, and the following decreasing order of adsorbability was indicated: free kitol, free vitamin A, kitol esters, vitamin A esters, anhydrovitamin A. Working on a 20–25 g. scale, fractions showing $E_{1\text{ cm.}}^{1\%}$ about 160 at 295 m μ . were regularly obtained, but they were not free from vitamin A. One such fraction was chromatographed

on weakened alumina (Spence, Grade 0, stirred with 10% (w/w) water). A fraction containing practically all the vitamin A was quickly obtained when the column was developed with light petroleum and was followed by a fraction showing $E_{1\text{ cm.}}^{1\%}$ 367.5 at 286 m μ . and a further fraction exhibiting $E_{1\text{ cm.}}^{1\%}$ 250 at 280 m μ .

Fig. 4. Ultraviolet absorption spectrum of portion of whale-liver oil insoluble in 80% (v/v) aqueous ethanol. Unsaponifiable fraction chromatographed, strongly adsorbed zone at top of column; $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) 588 in cyclohexane.

Attempts to crystallize kitol esters from crude preparations in acetone at low temperatures gave products of $\lambda_{\max}$ 280–286 m μ ., $E_{1\text{ cm.}}^{1\%}$ 160. Chromatography on '5% weakened' alumina gave two fractions $E_{1\text{ cm.}}^{1\%}$ 168 and 349.2, respectively.

Chromatography of cold acetone extract of frozen sperm-whale liver

A portion of oil (4.18 g.), dissolved in light petroleum (25 ml.), was put through a column of full-strength alumina and developed first with light petroleum (1 l.) and then with light petroleum containing increasing amounts of acetone. The eluates (1–7) were examined spectroscopically with the following results: (1) Light brown oil, discarded. (2) $\lambda_{\max}$ 328 m μ ., $E_{1\text{ cm.}}^{1\%}$ 254; some anhydrovitamin A was present. (3) $\lambda_{\max}$ 328 m μ ., $E_{1\text{ cm.}}^{1\%}$ 282; absorption curve practically normal for vitamin A. (4) $\lambda_{\max}$ 290–295 m μ ., $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) 253, gave no vitamin A band at 620 m μ . in the colour test. (5) Contained some vitamin A alcohol, $E_{1\text{ cm.}}^{1\%}$ (328 m μ .) 82. (6) $\lambda_{\max}$ 328 m μ ., $E_{1\text{ cm.}}^{1\%}$ 152, practically normal vitamin A curve (free alcohol). (7) Brown portion of column cut out, eluted with ether; fraction was mainly kitol (free), $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) 152.

Fraction 4 was rechromatographed on full-strength alumina and developed with light petroleum. Two main zones appeared and three portions were cut out from the middle of the lower zone. All three were eluted with ether. The ether

Fig. 5. Chromatography of phospholipin-free, neutral, sperm-whale-liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction $E_{1\text{ cm.}}^{1\%}$ (328 $m\mu.$) 305 (gross), 280 (corrected) [or 112.8 (corrected) with respect to oil]; solvent, cyclohexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

Fig. 6. Ultraviolet absorption spectrum of kitol ester fraction, 0.0013% in cyclohexane, 1 cm. $E_{1\text{ cm.}}^{1\%}$ (286 $m\mu.$) 346.

was removed and the weighed residues were tested in cyclohexane. The absorption curves were very flat over the range 286–290 $m\mu.$ and $E_{1\text{ cm.}}^{1\%}$ values of 166, 343 and 353 were obtained.

A large number of experiments on sperm-whale-liver oil indicated that in seeking to prepare pure kitol esters it is

desirable to use material free from phospholipins or free fatty acids. The following method is perhaps the simplest: To sperm-whale-liver oil acetone is added and, after allowing phospholipins to settle out, the solution is filtered through a bed of kieselguhr mixed with solid NaHCO_3 (used as a fine powder). The filtrate is diluted with water and extracted with light petroleum, washed with water, dried over Na_2SO_4 , filtered and the solvent evaporated. The oil (approx. 10 g.) is dissolved in light petroleum and chromatographed on alumina weakened by addition of 10% (w/w) of water. Light petroleum is used at first to develop the chromatogram (Fig. 5). The eluate is periodically tested with the SbCl_5 reagent. When the vitamin A colour test becomes weak, development is continued with light petroleum containing 5% ether (v/v). When kitol esters begin to be eluted a purple colour will be obtained with the SbCl_5 reagent. In two such experiments fractions showing $E_{1\text{ cm.}}^{1\%}$ (295 $m\mu.$) 346 (Fig. 6) were obtained weighing 0.85 and 0.52 g. respectively.

The best preparations of kitol (esterified) were all in agreement with the figure of $E_{1\text{ cm.}}^{1\%}$ about 350 as the highest attainable.

Saponification of kitol esters

The results of attempts to prepare pure kitol by saponifying the esters and extracting the unsaponifiable fraction were erratic and rather disappointing. Thus one ester preparation

Fig. 7. Ultraviolet absorption spectrum of kitol ester fraction, unsaponifiable matter after two treatments with alkali; $E_{1\text{ cm.}}^{1\%}$ (286 $m\mu.$) 230.

showing $\lambda_{\text{max.}}$ 286 $m\mu.$, $E_{1\text{ cm.}}^{1\%}$ 350 gave 54.8% of unsaponifiable matter with $\lambda_{\text{max.}}$ 280 $m\mu.$, $E_{1\text{ cm.}}^{1\%}$ 156, but the kitol had undoubtedly decomposed. The recovered acid amounted to 43% of the material used. Another good ester preparation, left to stand in the cold with ethanolic KOH for 48 hr., resisted saponification. On treatment with ethanolic KOH at the boiling point in a current of N_2 , the $E_{1\text{ cm.}}^{1\%}$ value of the unsaponifiable fraction rose to 410, but fell to 230 ($\lambda_{\text{max.}}$ 280 $m\mu.$) after retreatment with alkali for a longer time on the steam bath (Fig. 7). The recovered acids (33%) had a mean mol. wt. of 284.

With another preparation the total recovered fatty acids accounted for 48.3 % of the kitol ester fraction. The following illustrates some of the later experiments: 0.771 g. kitol ester fraction, $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) 346 was dissolved in ethanol (10 ml.) and 40 % aqueous KOH (4 ml.) was added. A little quinol was used as antioxidant. After boiling on the water bath until the solution was quite clear, the unsaponified material was extracted in the usual way and the fatty acids recovered from the soaps. The $E_{1\text{ cm.}}^{1\%}$ value at 285 m μ . had risen to 432 and 0.296 g. of acids was recovered. Resaponification with sodium ethoxide (boiling for 15 min.) raised the $E_{1\text{ cm.}}^{1\%}$ value of the non-saponifiable material to 620 and gave a further 0.125 g. of acids.

If kitol has a mol. wt. of 572 and an $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) of 700, then an $E_{1\text{ cm.}}^{1\%}$ value of 350 for mixed kitol esters implies that these are diesters of acids with an average mol. wt. of about 304.

Pyrogenic decomposition of kitol esters

When heated carefully, kitol esters darken in colour, and the product shows weakly a maximum at 620 m μ . in the SbCl_3 colour test. Small quantities (0.02 g.) heated electrically in capillary tubes showed no decomposition at temperatures below 200°. From 230 to 250° (4–5 min.) faint positive tests were obtained (SbCl_3 max. 612–620 m μ .), but the yield of vitamin A from the pyrogenic decomposition of the esters was poor. The special conditions of molecular distillation are clearly needed for good yields.

Administration of kitol esters to the avitaminotic rat

When given as 0.12 g. in 1 ml. of coconut oil spread over 2 days and administered by stomach tube, kitol esters were neither converted into vitamin A nor appreciably stored in the liver 40 hr. after the second dose.

A chromatographic method for the determination of vitamin A in whale-liver oils

The experiments leading to the isolation of a kitol ester fraction suggested a new approach to the analytical problem. The matter has a somewhat complicated history. At an early period in the history of the use of whale-liver oils as a source of vitamin A a commercial concentrate was studied (Edisbury *et al.* 1935, 1937) which exhibited anomalous ultra-violet absorption (λ_{max} about 295–305 m μ .) and an anomalous colour test as compared with fish-liver

oils. It was found empirically that by measuring $E_{1\text{ cm.}}^{1\%}$ (328 m μ .) and multiplying by 1200, estimates of potency in reasonably good agreement with the results of bioassays were obtained.

With the resumption of whaling after the end of the War it became necessary to determine vitamin A in crude oils extracted on board the factory ships. It speedily emerged in these laboratories, and in those of Lever Brothers and Unilever Ltd. at Port Sunlight, that neither the colour test nor the ultra-violet absorption was anomalous in a constant fashion, and the basis for the empirical use of a conversion factor of 1200 instead of 1600 (widely used for fish-liver oils) disappeared. The correction procedure of Morton & Stubbs (1946) was often stretched beyond the limits of its utility by the magnitude of the irrelevant absorption. It had also become clear that the factor of 1600 was itself a reflexion of the fact that, on the average, fish-liver oils exhibited some 11 % or so of 'irrelevant' absorption and that a factor of 1800 was appropriate to preparations in which vitamin A was the only significantly absorbing entity (Morton & Stubbs, 1947). The problem of analysing whale-liver oils thus resolved itself into the task of obtaining all the vitamin in a fraction (or fractions) free from other substances absorbing near 328 m μ . The following experiments indicate how the problem has been solved.

Sperm-whale-liver oil (1.713 g.) was dissolved in 15 ml. light petroleum in a small conical flask and powdered NaHCO_3 (1 g.) added, and the mixture thoroughly shaken. Weakened alumina was prepared by placing aluminium oxide (50 g., Spence, Grade 0) in a mortar, covering with light petroleum and adding water (5 ml.). The mixture was stirred thoroughly with a pestle and used to fill a tube 44 × 1 cm. A layer (1.5 cm. high) of powdered NaHCO_3 was put on top of the column. The oil in light petroleum was then poured on to the column without disturbing the sediment. The residual contents of the conical flask were washed with light petroleum several times and the solution poured on to the column. Light petroleum was used to develop the chromatogram. A light yellow band travelled down the column and the eluate was collected in 20 ml. portions, a drop from each of which was tested with the SbCl_3 reagent. The portions were sorted out into three groups: (A) those giving a normal vitamin A colour test, (B) those giving a purple colour and, finally, (C) those obtained only when the developing solvent contained ether (10 or 20 % by vol. in

Table 3. *Chromatographic separation of constituents of sperm-whale-liver oil*

Fractions	Wt. recovered (g.)	λ_{max} (m μ .)	(Wt. of oil, 1.713 g.)		$E_{1\text{ cm.}}^{1\%}$ with respect to oil	SbCl_3 colour test (λ_{max})
			$E_{1\text{ cm.}}^{1\%}$			
A	0.517	328	(328 m μ .) 316 gross 280 corr.		(328 m μ .) 96 gross 84 corr.	620 m μ . (blue)
B	0.364	300	(300 m μ .) 218.5 gross (328 m μ .) 34 corr. (286 m μ .) 233 corr.		(328 m μ .) 7.2 corr. (286 m μ .) 49.4	(purple), 495 m μ ., 505 m μ ., 580 m μ .
C	0.064	280	(280 m μ .) 38 gross		—	(Red)

light petroleum). The solvent was removed from the three fractions and the residues weighed and subjected to spectroscopic analysis (Table 3).

It is evident from Table 3 that fraction *A* contained most of the vitamin A and was easily analysed, *B* contained most of the kitol, and *C* consisted mainly of decomposition products. A further experiment was carried out to try to improve the separation.

Sperm-liver oil (0.998 g.) and NaHCO_3 (0.5 g.) were shaken with light petroleum and the solution chromatographed on weakened alumina as before. The eluate was collected in 5 ml. portions and those which gave a normal vitamin A blue with the SbCl_3 reagent were bulked, the solvent removed and the residue weighed (0.282₄ g.). This was made up to volume with cyclohexane and a measured portion was diluted for spectrophotometry. The absorption maximum occurred at 328 m μ . with $E_{1\text{ cm.}}^{1\%}$ 100.8 (gross) calculated on the weight

Fig. 8. Chromatographic analysis of sperm-whale-liver oil. Ultraviolet absorption spectrum of vitamin A ester fraction; $E_{1\text{ cm.}}^{1\%}$ (328 m μ .) 100.8 (gross), 94.4 (corrected) with respect to oil; solvent, cyclohexane. Upper curve, observed; lower curve, corrected; broken line, subtraction curve.

of oil used (Fig. 8). This value was reduced to 94.4 on applying the three-point correction procedure for irrelevant absorption. The previous experiment gave $84 + 7.2 = 91.2$, but the vitamin was not all in the first fraction. It will be seen that by collecting the eluate in 5 ml. portions, one drop of each of which is tested with SbCl_3 , a very clear-cut separation can be achieved.

The procedure needs, however, to be subjected to a more severe test. Accordingly, a sample of low-potency oil of inferior quality was tested.

Crude whale-liver oil (0.2665 g.) in light petroleum (20 ml.) was chromatographed on a column of weakened alumina 15×1 cm. and developed with light petroleum. The eluate was collected in 5 ml. portions. The first few portions contained anhydrovitamin A (pale greenish yellow; blue colour with SbCl_3). The next four portions gave no colour with the

SbCl_3 reagent and were discarded. Subsequent portions contained vitamin A (esters) giving a normal colour test. Elution was continued until the solution was no longer chromogenic towards SbCl_3 . The column was then eluted with ether. The vitamin A fraction was made up to 50 ml. with light petroleum and a measured portion diluted with cyclohexane so that the concentration was 0.0533% with respect to the original oil. It gave λ_{max} 328 m μ ., $E_{1\text{ cm.}}^{1\%}$ 8.74 gross, 8.4 (corr.). The ether eluate was made up to 100 ml. with light petroleum, and a measured portion diluted with cyclohexane to 0.02665% with respect to the original oil. It gave $E_{1\text{ cm.}}^{1\%}$ (286 m μ .) 12.5 and contained kitol esters together with decomposition products, but no vitamin A was detectable. (If at any time the percolation of solvent through the column becomes too slow, it is advisable to stir the adsorbent at the top with a glass rod.)

To test the method further a more complete analysis was made on another sample of crude oil. Crude whale-liver oil (0.2 g.) in light petroleum (20 ml.) was poured on a column (15×1 cm.) of weakened alumina, and the chromatogram developed with light petroleum followed by the same solvent containing increasing proportions of ether. The percolate was collected in 5 ml. portions, 1 drop of each being tested with the SbCl_3 reagent.

The following fractions were obtained: (1) Anhydro-vitamin A, discarded. (2) Vitamin A esters; 180 ml. of eluate λ_{max} 328 m μ ., $E_{1\text{ cm.}}^{1\%}$ 14.6 (gross), 14.14 (corrected); *E* values calculated on weight of oil taken. (3) Kitol ester fraction; elution complete after using light petroleum containing 4, 8 and 12% ether. λ_{max} 290 m μ ., $E_{1\text{ cm.}}^{1\%}$ 8.125. (4) Fraction containing free vitamin A and some free kitol; λ_{max} 326 m μ ., $E_{1\text{ cm.}}^{1\%}$ 4.22 (gross), 3.02 (corr.); elution completed with 12, 16 and 20% ether in petrol. (5) Fraction eluted with ether; no vitamin A present as judged by colour test.

For this oil $E_{1\text{ cm.}}^{1\%}$ (328 m μ .; gross) was 28.7 and the net contribution due to vitamin A was $14.14 + 3.02 = 17.16$. The simple correction procedure of Morton & Stubbs (1946) gave 15.8—a figure which is significantly low.

Similar experiments have led to the following standard procedure.

Analysis of commercial whale-liver oil

Adsorbent. Alumina (Spence, Grade 0) for adsorption (100 g.) is made into a slurry with light petroleum in a glass mortar, and water (10 ml.) is added and the whole mixed with a pestle for 5 min. The slurry is then poured into an adsorption tube and allowed to settle. The weakened adsorbent should be used the same day.

Column. The column of weakened alumina should be 12×1 cm. if the weight of oil to be chromatographed is 0.2 g. The sample is dissolved in light petroleum (25 ml.) and poured on to the column. A large bottle (15–20 l.) is fitted with a rubber bung with two holes, through which tubes, each with a tap, are inserted; one is connected by a rubber tube to a bicycle pump and the other to a short piece of glass tubing in a rubber bung fitting the top of the

adsorption apparatus. Passage of solvent through the column is thus hastened by pressure from above, and percolation may be quite rapid (approx. 2 ml./min.). There should be room for at least 25 ml. of solvent above the absorbent.

Development. The chromatogram is developed first with light petroleum and the percolate collected in small test tubes graduated at 5 ml. One drop from each portion is tested with 0.5 ml. of SbCl_3 reagent. The first few portions will give no colour and may be discarded. Then anhydrovitamin A will appear in the eluate; it gives a blue colour with the SbCl_3 reagent and, after a volume of 35–40 ml. has passed through the column, no colour will appear. (The volume will, of course, vary somewhat from sample to sample.) Continued development with light petroleum results in the appearance of vitamin A esters in the eluate. The intensity of the blue colour obtained with one drop of successive portions of percolate will at first increase and finally decrease until no colour is given. By this time, 100–200 ml. of liquid will have passed through the column. Development is continued at first with light petroleum containing 4% ether (by vol.) and then with 8 and with 12% ether-petroleum mixtures, usually 50 ml. portions at each strength. The percolate at this stage gives a purple colour with the SbCl_3 reagent and exhibits an ultraviolet absorption maximum near 285–290 $\text{m}\mu$. A small volume of eluate then appears which is practically non-chromogenic towards SbCl_3 . Development with ether-petroleum mixtures (12, 16, 20% by vol.) is continued, and fractions are obtained giving blue and purple colours with SbCl_3 , indicating free vitamin A and free kitol respectively. The 5 ml. portions of eluate are grouped and the separate fractions each made up to a definite volume and examined by photoelectric spectrophotometry.

Example. $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 60.1 on the whole oil.
0.125 g. examined as above:

- (i) Anhydrovitamin A fraction $E_{1\text{ cm.}}^{1\%}$ (370 $\text{m}\mu$.) 1.6.
- (ii) Vitamin A ester fraction, made up to 200 ml., diluted 5 ml./20 ml. cyclohexane. $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 37.9, no correction needed.
- (iii) Kitol ester fraction, $\lambda_{\text{max.}}$ 285–290 $\text{m}\mu$. $E_{1\text{ cm.}}^{1\%}$ 24.35.
- (iv) Free vitamin A fraction, $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) approx. 2.2 corr.

Total $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 40 (i.e. 37.9 + 2.1).

Estimated potency $40 \times 1800 = 72,000$ i.u./g.

Separation of vitamin A from whale-liver non-saponifiable matter by means of 50% ethanol

By washing the unsaponifiable fraction of whale-liver oil with 50% (v/v) aqueous ethanol, free vitamin A is preferentially dissolved. Under certain circumstances this forms the basis of a useful analytical procedure.

Example. A commercial whale-liver oil concentrate (0.5 g.; $E_{1\text{ cm.}}^{1\%}$ (328 $\text{m}\mu$.) 210 gross) was saponified, and the unsaponifiable fraction showed $E_{1\text{ cm.}}^{1\%}$ 212.6 at 326 $\text{m}\mu$. (calculated on the wt. of raw material). The absorption curve was, however, anomalous and there was evidence of considerable irrelevant absorption. The unsaponifiable matter was shaken well with 50% ethanol (100 ml.), and the supernatant liquid filtered carefully through a small bed of kieselguhr (filter aid). Six further washings (2 x 100 ml. and 4 x 50 ml.) were filtered in the same way. The combined filtrate was made up to 500 ml. (A). The flask and the filter aid were then washed repeatedly with small quantities of redistilled ether. The solvent was removed and the extract dried by treatment with ethanol in a current of N_2 on the water bath; the residue was dissolved in ethanol and the volume made up to 100 ml. (B).

A measured volume of solution A was diluted with ethanol and showed $\lambda_{\text{max.}}$ 326 $\text{m}\mu$, $E_{1\text{ cm.}}^{1\%}$ 135.8 (corrected by the Morton & Stubbs (1946) method). Solution B, suitably diluted, gave $\lambda_{\text{max.}}$ 287 $\text{m}\mu$, $E_{1\text{ cm.}}^{1\%}$ 85 (E values calculated with respect to the original oil; see Fig. 9).

Fig. 9. Ultraviolet absorption spectrum. — Portion of whale-liver oil unsaponifiable material soluble in 50% (v/v) aqueous ethanol; $E_{1\text{ cm.}}^{1\%}$ (326 $\text{m}\mu$.) 164 (gross), 135.8 (corrected), calculated for original oil; solvent, ethanol. ---- Portion of unsaponifiable material insoluble in 50% ethanol; $E_{1\text{ cm.}}^{1\%}$ (287 $\text{m}\mu$.) 85; solvent ethanol.

Further trials showed that most, but not all, of the irrelevant absorption can be eliminated from whale-liver oil unsaponifiable fractions by extraction with 50% aqueous ethanol. The kitol fraction is relatively insoluble in this medium. Judged, however, by the strictest standards, the method is imperfect, because when an oil is contaminated with artifacts, as well as with kitol, a little extraneous

material absorbing at 326 m μ . is liable to accompany the vitamin A alcohol. The test, however, often affords valuable confirmation (see Table 4).

Table 4. *Correction of absorption of a whale-liver oil concentrate*

(Concentrate had $E_{1\text{cm}}^{1\%}$ (328 m μ .) 210 (gross))		
Concentrate	E (corr.)	Vitamin A
	141.4	* Assuming it to be all esterified
	128.8	* Assuming it to be free
Unsaponifiable extract	130	* Assuming it to be free
50% ethanol extract	135.8	* Assuming it to be free

* The absorption curves for vitamin A alcohol and esters are not quite identical.

DISCUSSION

Numerous preparations from samples of whale-liver oil have been obtained showing an absorption maximum at 285–295 m μ . with $E_{1\text{cm}}^{1\%}$ 346–360, and further chromatography has failed to raise the intensity of absorption. The preparations were practically free from glycerides or vitamin A. They underwent saponification with difficulty, but finally yielded approximately half their weight as recovered fatty acids after extracting the acidified soaps with ether. The unsaponifiable material tended to decompose with consequent loss of absorption intensity, but in one case an $E_{1\text{cm}}^{1\%}$ (286 m μ .) value of 620 was obtained when quinol was used as antioxidant during the saponification. Kitol, which has been reported as possessing an $E_{1\text{cm}}^{1\%}$ value of 700 when obtained as freshly recrystallized material, is not very stable, even in the solid state in sealed ampoules; it does not withstand chromatography without decomposition and it is unstable towards alkali. If it is re-esterified, the esters are sometimes recrystallizable, but *cis-trans* isomerism may bring in complications.

The 'purified' kitol preparations of $E_{1\text{cm}}^{1\%}$ 350 are clearly mixed diesters containing an assortment of higher fatty acids of molecular weights exceeding 280. The preparation of such kitol esters appears to rule out the possibility that kitol is an artifact, and to show that kitol esters are present in whale liver. If the samples of blue- and sperm-whale livers were representative, kitol esters are quantitatively much more significant in sperm whales than in blue whales. Too little, however, is known about variations between individual whales of either species to permit this observation to be generalized.

The fact that kitol is a di(vitamin A) is fully established, and from its absorption spectrum it must have a chain (or two chains) of four conjugated double bonds instead of the five conjugated double bonds of vitamin A. The molecular extinction coefficient of kitol is about 41,000 and that of vitamin A about 50,000.

Gridgeman, Savage & Gibson (1948), working at the Port Sunlight laboratories of Lever Bros. and Unilever Ltd., have for some time been engaged upon the problem of determining vitamin A in whale-liver oils. They have worked out an excellent method for the separation of vitamin A alcohol from unsaponifiable extracts by chromatography, and we are indebted to Messrs Lever Bros. for information and advice over a long period.

Comparing the method we have described with the procedure based on preliminary saponification it seems likely: (a) that for oils in which much of the vitamin A is free there are advantages to be gained from completing the saponification in aiming at an estimate of the vitamin A content at the time of the test; (b) that for oils in which most of the vitamin A is esterified, direct chromatography is preferable in minimizing losses and in speed of operation; (c) that a determination of free and esterified vitamin A gives some indication of the probable stability of the oil, as it is the free vitamin A which forms anhydro-vitamin A and probably some artifacts.

SUMMARY

1. The acetone-soluble lipid from a sample of frozen blue-whale liver contained vitamin A ($E_{1\text{cm}}^{1\%}$ (328 m μ .) 36.7) with relatively little kitol. A similar extract from frozen sperm-whale liver was richer in vitamin A and kitol (16 and approx. 20 %, respectively, as esters).
2. The fractionation of whale-liver oils has been attempted in various ways. Free vitamin A is extracted by means of 50 % aqueous ethanol, and vitamin A esters are separable from kitol esters by means of 80 % aqueous ethanol.
3. Chromatography of whale-liver oil on alumina (weakened by addition of 10 % (w/w) water) effects a clean separation of vitamin A (esterified) from kitol (esterified). Kitol ester fractions showing $E_{1\text{cm}}^{1\%}$ approx. 350 and approx. 170 at 286 m μ . were regularly obtained.
4. Kitol esters are difficult to saponify, and the free kitol tends to be decomposed unless precautions are taken. They contain, however, about half their weight of kitol. $E_{1\text{cm}}^{1\%}$ (286 m μ .) values of about 620 have been obtained for free kitol.
5. Kitol esters fed to rats are neither converted to vitamin A nor stored in the liver.
6. A chromatographic method for the determination of vitamin A in unsaponified whale-liver oils has been described and tested.
7. A fairly good separation of kitol from vitamin A in whale-liver unsaponifiable fractions can be effected by extracting the vitamin A with 50 % aqueous ethanol, but the volume used limits the method to analytical work.

8. Preliminary work on whale-liver phospholipins suggests that kitol is not present in the true acetone-insoluble lipid, but that it may occur in more than one type of combination.

9. Vitamin A and possibly kitol is present in fin-whale milk.

A small sample of kitol was kindly placed at our disposal by Dr Baxter of Distillation Products Inc. We are indebted to the Ministry of Food and the Medical Research Council for grants in aid of the work. In obtaining material we have had most friendly help from the whaling industry and many scientists engaged in it both in this country and abroad.

REFERENCES

- Baxter, J. G., Clough, F. B., Kascher, H. M. & Robeson, C. D. (1947). *Science*, **105**, 436.
Chevallier, A., Manuel, S. & Faubert, P. (1941). *Trav. Soc. Chim. Biol.* **23**, 1429.
Edisbury, J. R., Morgan, R. S. & Morton, R. A. (1935). *Biochem. J.* **31**, 258.
Edisbury, J. R., Morton, R. A., Pritchard, H. & Wilkinson, H. (1937). *Biochem. J.* **31**, 258.
Embree, N. D. & Shantz, E. M. (1943). *J. Amer. chem. Soc.* **65**, 906, 910.
Gridgeman, N. T., Savage, J. P. & Gibson, G. P. (1948). *Analyst*, **73**, 662.
Hickman, K. C. D. (1943). *Ann. Rev. Biochem.* **12**, 317.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Morton, R. A. & Stubbs, A. L. (1947). *Biochem. J.* **41**, 525.

Studies in Vitamin A

13. THE ALLEGED FORMATION OF VITAMIN A FROM β -CAROTENE TREATED WITH IODINATED CASEIN

By H. R. CAMA AND T. W. GOODWIN, *Department of Biochemistry, University of Liverpool*

(Received 19 April 1949)

Kaplansky & Balaba (1946) claimed that β -carotene in colloidal solution when incubated with either thyroglobulin or iodinated casein was converted into vitamin A in good yield; a typical experiment resulted apparently in the production of 60 μ g. of vitamin A from 150 μ g. of β -carotene. If it be true that thyro-active compounds can effect such a conversion, the finding is of major importance to the biochemistry of carotene and vitamin A. An investigation of Kaplansky & Balaba's claim was therefore undertaken. In the first part of the work the experimental procedure of Kaplansky & Balaba was followed as closely as possible, but when efforts to produce vitamin A failed, the conditions were then varied. A short report of the results of this work has already appeared (Cama & Goodwin, 1948).

EXPERIMENTAL

Preparation of iodinated casein. NaHCO_3 (3.6 g.) was added to skimmed milk (500 ml.) freed from fat by repeated centrifugation, and the liquid heated to 40°. Small portions of I_2 were added with constant stirring until a slight excess remained at the bottom of the beaker (about 8.0 g. of I_2 were required). The temperature was then raised to 65° for 24 hr., the liquid being constantly stirred. After cooling, the intensely yellow solution was filtered and the filtrate brought to pH 4.5 with acetic acid. The precipitated iodinated casein was washed, dissolved in very dilute alkali and reprecipitated with acetic acid. This process was repeated three times. The I_2 content of the product, determined according to the

micro method of Groák (1926) was 8.45–8.67%. Kaplansky & Balaba (1946) report 0.85%, but this appears to be a mistake.

Preparation of colloidal solutions of β -carotene. Crystalline β -carotene (approx. 3 mg.) was dissolved in a small volume of freshly redistilled diethyl ether (20 ml.) and 96% (v/v) ethanol added (5 ml.). The ether was removed by distillation on a water bath at 50–60°; the distillation was continued until a test portion of the distillate no longer separated into two layers after the addition of a small volume of water. The ethanol solution of β -carotene was then diluted with distilled water and rapidly filtered; the colloidal solution was placed in a boiling water bath to remove the ethanol and any residual ether. The remaining concentrated colloidal solution was filtered again, diluted with water to approximately the desired concentration and used without delay.

Incubation of solutions. The details of Kaplansky & Balaba's (1946) method are as follows: To a known volume of colloidal solution containing 150 μ g. β -carotene is added iodinated casein (30 μ g.) dissolved in weak alkali; the reaction mixture, after being adjusted to pH 7.3 with acetic acid, is incubated for 2 hr. at 37°; controls consisting of β -carotene solutions only or β -carotene solution + boiled iodinated casein adjusted to pH 7.3 are treated in the same way.

In the first stages of the present investigation the above procedure was followed as closely as possible. (Kaplansky & Balaba did not specify the concentration of the weak alkali or the acetic acid; we used 0.01 N- NaHCO_3 and 0.01 N-HOAc.) The procedure was later modified by altering (a) the pH (by the use of phosphate buffers), (b) the incubation times, and (c) the amounts of substrate and 'enzyme', and (d) by using desiccated thyroid or minced fresh thyroids instead of iodinated casein.