the detection of D-arabinose and D-arabonic acid in heated solutions of 2-keto-D-gluconic acid, Steinberg states that neither D-arabinose nor D-arabonic acid is utilized by *A. niger* for purposes of growth, and that the same is true of D-xylulose. Hence, it would appear that compounds other than these are formed in the thermal decomposition of 2-keto-D-gluconic acid, and it may well be that ribulose is a useful source of carbon for *A. niger*.

The scheme presented in Fig. 1 symbolizes our idea of the way in which calcium tartronate could arise from calcium 2-ketogluconate in presence of dilute alkali. A sequence of reactions leading to D-glyceraldehyde and the semialdehyde of tartronic acid followed by a Cannizzaro reaction between these two aldehydes would seem to constitute a reasonable explanation of the observed facts. D-Ribulose (I) may be presumed to arise by transformation of D-arabinose (II) or by decarboxylation of the hypothetical intermediate (III) which is 3-keto-D-gluconic acid. Our failure to detect ribose suggests that ribulose arises mainly by a mechanism other than isomerization of arabinose, since accumulation of ribose in appreciable quantity would be expected if this route was taken. In this connexion it is relevant to note that Gross & Lewis (1931) treated xylose with lime-water at 32° and detected the presence of D-xylulose and D-lyxose in equivalent quantities, at final equilibrium.

SUMMARY

1. The calcium salts of 2-keto-D-gluconic and tartronic acids were isolated from cultures of *Acetobacter acetosum* which had been grown in a medium containing glucose and calcium carbonate.

2. When an aqueous solution of calcium 2-keto-D-gluconate was heated at 100° for 20 min. with calcium hydroxide in an atmosphere of nitrogen,

calcium tartronate was formed and was isolated in small yield from the mixture of products. By paper chromatographic analysis it was demonstrated that arabinose and ribulose had been formed also in this reaction.

3. A scheme of reactions to account for these findings is presented.

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Some Minor Constituents of Liver Oils

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Minor constituents of liver oils, such as carotenoids, vitamins A and D and provitamins D, have attracted much study, but it is not unreasonable to assume that other, and possibly important, substances are present in small amount and have yet to be identified. The nature of such compounds might throw light on metabolic pathways of vitamins or sterols.

In order to isolate minor constituents, it may be

necessary to use large amounts of the starting material and to carry out lengthy manipulations with the attendant risk of producing artifacts, particularly from vitamin A. The greater the quantity of material used initially, the more serious is the risk of contamination; thus, liver tissues worked up in a commercial plant may yield lipid extracts slightly contaminated with impurities from solvents and these may accumulate in indi-

vidual chromatographic fractions. On the other hand, if after experience has been gained of such artifacts as can be met with in working up normal tissue, something new is encountered in extracts similarly prepared from abnormal tissue, it may well be significant.

In the present work on liver oils and their unsaponifiable fractions the method of separation has been chromatography on alumina, with ultraviolet and visible spectrophotometry as the main analytical tool.

We began with a study of a fish-liver oil rich in vitamin A, to see whether any minor, structurally related congener of vitamin A could be detected. Although most of the vitamin A could be concentrated into one chromatographic fraction, the spectra of most of the other fractions were affected by the presence of vitamin A artifacts. As some of these might have been produced in extracting the oil from liver, the next step was to use liver tissue and to carry out the extraction under mild conditions. Frozen fin-whale liver was chosen as the raw material, but again artifacts mainly derived from vitamin A and kitol reduced the chances of isolating minor constituents. As the whale liver had necessarily been stored for a long time, fresh livers from sheep and ox were studied. Some gain accrued from using fresh tissue, but the presence of carotenoids tended to mask minor constituents with similar adsorptive properties and also turned out to be an additional source of artifacts. A large batch of ox-liver lipid (obtained as a pharmaceutical by-product) was next studied but here contamination was the main difficulty.

By using tissue from horses killed by the knacker, it was possible to combine the advantages of having fresh tissue with minimal interference from vitamin A and carotenoids, because the livers were very low in vitamin A. These favourable circumstances had, however, to be balanced against the risk that any minor constituents isolated from the livers of vitamin A-depleted animals might differ from those present in normal livers. It was therefore necessary to examine tissue from well-fed healthy horses slaughtered for meat. Kidney and spleen tissues were also studied, partly because they contain little vitamin A to produce interfering artifacts.

EXPERIMENTAL

Argentine shark-liver oil

This oil (25 g.) was chromatographed directly on alumina. Light petroleum carried through 70% of the vitamin A (i.e. about 1.5×10^6 i.u.) with the first 2.67 g. of oil. Any vitamin A congeners present could not be separated. Chromatography of the oil rather than the unsaponifiable fraction results in less artifact formation but separations are not very sharp. The results of several experiments were

not promising for the isolation of minor constituents. Some interesting pointers however were the presence of (a) a minute amount of an unsaponifiable constituent with absorption maxima at 255, 261, 269, 272 and 316 m μ ., (b) a very small amount of another unsaponifiable constituent with maxima at 283, 287 and 297 m μ ., and (c) a third unsaponifiable material with λ_{max} 276 m μ .

Fin-whale liver

A portion of fin-whale liver brought frozen to England on the Factory Ship *Balaena* was kept in cold store until used. The lipid, obtained by ether extraction after grinding with sharp sand and anhydrous Na_2SO_4 , showed general absorption with inflexions at 270 and 320 m μ . The vitamin A content was moderate (about 100 i.u./g. liver).

The oil (4 g.) was chromatographed on weakened alumina (Spence Grade 0 alumina plus 5%, v/w, water). A main vitamin A ester fraction eluted with 5% ether-light petroleum showed λ_{max} 300 and 321 m μ ., $E_{1\text{cm}}^{1\%}$ 0.88 calculated on the weight of crude oil. A fraction showing λ_{max} 275 m μ ., $E_{1\text{cm}}^{1\%}$ 0.22 (calculated on the crude oil) was then eluted (10% ether-light petroleum) and was followed by a fraction containing free vitamin A alcohol (eluted with 50% ether-light petroleum). Further experiments on a larger scale failed to separate the substance with λ_{max} 275 m μ completely from vitamin A.

Chromatography (on Spence grade 0 alumina plus 5%, v/w, water), of the unsaponifiable fraction after removal of some cholesterol by crystallization from light petroleum, gave three small (20–30 mg.) fractions: (a) a weakly adsorbed waxy solid with λ_{max} 255 m μ ., $E_{1\text{cm}}^{1\%}$ 21, eluted by light petroleum; (b) a pale yellow resin with well-defined maxima of low intensity at 268 and 328 m μ . (eluted by 2%, v/v, ether-light petroleum; SbCl_3 colour test maximum anomalous, at 640–650 m μ .); (c) a rather similar fraction (also eluted by 2% ether-light petroleum) with λ_{max} 274 and 332 m μ ., the latter more intense at $E_{1\text{cm}}^{1\%}$ 3.8 (SbCl_3 λ_{max} 640–650 and 580 m μ .). The main bulk of the unsaponifiable matter (eluted by ether) showed λ_{max} 295 m μ . From this absorption curve, the SbCl_3 colour test and the appearance of the solid portion, the fraction contained kitol, free vitamin A and cholesterol.

From previous experience on fin-whale liver oil it was thought possible that the frozen liver might have yielded an oil suitable for the detection of minor constituents. In fact, the occurrence of fractions exhibiting unusual colour tests with the SbCl_3 reagent and abnormal ultraviolet spectra suggested that artifacts were produced with unusual readiness in material subjected to prolonged storage. The outcome of the tests is that such products together with kitol are a hindrance, but that nevertheless substances absorbing near 270 m μ . are worthy of further study.

Sheep liver

Small samples of fresh livers from healthy animals were obtained from the local abattoir. The lipid extracts (about 5% on wet weight of liver) showed $E_{1\text{cm}}^{1\%}$ 328 m μ . 9.7–15.3; the selective absorption was due mainly to vitamin A (600–1200 i.u./g. fresh liver), with little or no irrelevant absorption on the long-wave side of the maximum. Chromatography of a fresh portion of lipid gave a small fraction (eluted with light petroleum but following the normal vitamin A ester) with λ_{max} 270–272 m μ ., SbCl_3 λ_{max} .

595 m μ . The chromogen was probably derived from vitamin A and may have been an epoxide of the esterified vitamin. A preparation of unsaponifiable matter (about 0.66% on wet weight of liver) showed $E_{1\text{cm}}^{1\%}$ 326 m μ . about 117 with very little irrelevant absorption. Chromatography of the unsaponifiable fraction revealed no new constituent unambiguously; small amounts of artifacts interfered considerably.

In the course of further chromatography applied to the unsaponifiable fraction the presence of two carotenoid hydrocarbons, β -carotene (λ_{max} 445–446, 470–473 m μ .) and another substance not fully identified (λ_{max} 445, 468 and 500 m μ .) was established. A substance with λ_{max} 275 m μ . was present in the sterol fraction in very small amount. Other fractions showing selective absorption were probably artifacts.

It is interesting that carotene can be found in sheep liver fat although the body fat is very white. The high vitamin A content hampers the detection of minor constituents, but it is important not to disregard the possibility that such substances may be present in appreciable amounts.

Ox liver

Fresh tissue. Fresh liver tissue yielded 5.1% lipid showing inflexions at 270–275 m μ . ($E_{1\text{cm}}^{1\%}$ 3.0) and 320–330 m μ . ($E_{1\text{cm}}^{1\%}$ 2.4) consistent with about 130 i.u./g. liver of vitamin A. Chromatography on alumina gave a light petroleum eluate containing the vitamin A esters and showing a normal vitamin A spectrum, whilst the material eluted by acetone contained a quite small amount of free vitamin A but exhibited a marked inflexion near 270 m μ .

A second sample of tissue, from another liver, yielded 4.5% lipid, while the unsaponifiable fraction obtained after digesting the tissue with alkali showed λ_{max} 328 m μ ., $E_{1\text{cm}}^{1\%}$ 27, with evidence of moderately intense irrelevant absorption on the short-wave side of the peak. Chromatographic separation of this unsaponifiable material on weakened alumina resulted in the elution first (by light petroleum) of carotenoid (λ_{max} 424, 442, 470 m μ .) followed by materials showing λ_{max} 447, 462, inflexion 500 m μ . and λ_{max} 443, 470 and 500 m μ ., both eluted by 4% (v/v) ether–light petroleum. After the carotenoids, 4–8% (v/v) ether–light petroleum eluted fractions showing absorption maxima at 274 and 333 m μ . (565 m μ . in the SbCl₃ colour test). Vitamin A was then eluted, followed later by the sterols, as the eluting solvent was enriched in ether. The fractions showing two bands (274 and 333 m μ .) were interesting, but it was at this stage impossible to decide whether the selective absorption was due to an artifact or not.

Pharmaceutical by-product. A sample of ox liver lipid obtained as a by-product in preparing liver extracts, was kindly provided by Evans Medical Supplies. It was a pale yellow solid fat with a slight odour due to the presence of a small amount of preservative. The absorption curve of the unsaponifiable fraction (7.47 g.) showed a small maximum at 255 m μ . with inflexions at 295 and 330 m μ ., and carotenoid bands in the visible region. Only those fractions which differed from those obtained with fresh liver will be discussed. Chromatography of the unsaponifiable matter on weakened alumina (100 g. plus 5 ml. water) resulted in three fractions eluted with light petroleum and, in addition, well-marked zones on the column; the alumina was extruded and the sections were extracted separately. The first petroleum eluate (0.69 g.) was a liquid showing only weak ultraviolet absorption. The second fraction (0.13 g.)

exhibited an intense absorption band λ_{max} 256 ($E_{1\text{cm}}^{1\%}$ 675); and also peaks at 445 m μ . ($E_{1\text{cm}}^{1\%}$ 68) and 472 m μ . due to carotene.

The first fraction (i.e. least adsorbed) from the extruded column (0.36 g.) showed λ_{max} 271 and 298 m μ . and fluoresced strongly under ultraviolet illumination. The remaining fractions contained much vitamin A.

The fraction containing the blue fluorescent material was rechromatographed on alumina (weakened with water 5%, v/w). The light petroleum eluate (0.088 g.) was fluorescent and showed sharp absorption bands at 245, 271, 298, 308

Table 1. *Ultraviolet absorption of contaminant compared with that of C₁₉H₁₆O (Morice, 1951)*

Figures in italics refer to inflexions, those in parentheses are quoted from Morice (1951).

(Solvent, ethanol)			
λ_{max} (m μ .)	$E_{1\text{cm}}^{1\%}$	λ_{min} (m μ .)	$E_{1\text{cm}}^{1\%}$
348 (348)	170 (190)	332	147 (ca. 150)
310 (310)	928 (1021)	283	475 (ca. 500)
272 (272)	1075 (1180)	230	537 (ca. 500)
248	720		
222 (220.5)	2000 (2225)		
(Solvent, cyclohexane)			
355	117	325	134
343	156	303	775
309	852	282	512.5
300	792.5	232	647
265	925		
272	1062		
255	810		
246–248	800		
222	2075

Fig. 1. Absorption curve of the best preparation of Morice's compound obtained in the present work.

and 340 $\mu\mu$. The first extruded fraction (0.039 g.) showed $\lambda_{\max}$ 266 $\mu\mu$. ($E_{1\text{cm}}^{1\%}$ 91) and the remaining fractions had inflexions only.

Rechromatography on similar alumina of this fluorescent material gave one fraction (10 mg.) in which the main peak at 271 $\mu\mu$. had $E_{1\text{cm}}^{1\%}$ 595. Other peaks were shown at 298, 305 and 342 $\mu\mu$. This material, which was obtained in 1949 proved very stable and has recently been rechromatographed by one of us (R. A. M.) assisted by Mr R. H. Creed. The properties of the purest fraction (Table 1) agree with those of a compound isolated by Morice (1951) in the course of work on butter. The nature of the compound is discussed later (p. 280); it is almost certainly a laboratory contaminant or artifact.

Rechromatography of the extruded fraction with $\lambda_{\max}$ 266 $\mu\mu$. raised the $E_{1\text{cm}}^{1\%}$ value to 119.

Examination of the preservatives added to the liver fat by the manufacturers showed that they could not account for any of the absorbing substances. The general comment may be made that the presence of carotenoids, vitamin A and decomposition products makes ox liver fat not very suitable for the isolation of minor constituents.

Horse liver, kidney and spleen

Much work was carried out on horse tissue, and it will be convenient to consider the results on separate horses in succession. Some of the horses had very low liver reserves.

First horse liver. This sample of liver was obtained from a knacker's yard where horses are slaughtered because accident or old age has made them unfit to work. A trial on 25 g. of liver gave 0.8 g. lipid showing $\lambda_{\max}$ 263 $\mu\mu$. ($E_{1\text{cm}}^{1\%}$ 1.9) an inflexion near 330 $\mu\mu$. ($E_{1\text{cm}}^{1\%}$ 0.6) and a feeble maximum at 416 $\mu\mu$. in cyclohexane. The vitamin A content was quite low. A larger portion (300 g.) was subjected to alkali digestion to give 1.1 g. unsaponifiable material, showing a broad inflexion at 260–285 $\mu\mu$. and a weak vitamin A band which on correction for irrelevant absorption indicated 23 i.u./g. for the vitamin A content of the liver tissue.

After crystallizing and removing cholesterol from a methanol solution, the unsaponifiable fraction was chromatographed. A very small fraction with a single intense absorption peak at 271 $\mu\mu$. was eluted with 20% (v/v) ether–light petroleum.

Another portion (1450 g.) of the same liver was subjected to alkali digestion and 5.04 g. of unsaponifiable matter was obtained. A part (0.68 g.) of this was chromatographed in the same way to give, on elution with 20% (v/v) ether–light petroleum, immediately before vitamin A, 12 mg. of material with $\lambda_{\max}$ 271 $\mu\mu$., $E_{1\text{cm}}^{1\%}$ 106. The sterol fractions (more strongly held than vitamin A and eluted with 60% (v/v) ether–light petroleum) were contaminated with material showing $\lambda_{\max}$ 283 $\mu\mu$. A larger scale separation gave a smaller yield of this '271 $\mu\mu$. substance' than was anticipated from the pilot experiment.

Second horse liver. This material also came from the knacker's yard and the low vitamin A potency (about 5 i.u./g.) suggested a poor state of nutrition. The unsaponifiable fraction (0.2% of liver weight) showed a well-defined peak at about 270 $\mu\mu$. and an inflexion at 330 $\mu\mu$.; after removal of cholesterol by crystallization, chromatography of the residue gave a very small fraction with an intense peak at 270 $\mu\mu$.

Larger portions (6 kg. in all) were worked up to yield 17 g. of unsaponifiable material with $E_{1\text{cm}}^{1\%}$ 4.4–5.0 at 269–272 $\mu\mu$. After removing much sterol at 0° from methanol, the unsaponifiable matter was subjected (in portions) to chromatography as before. In each case a '270 $\mu\mu$. substance' was slightly more strongly held than carotene and was followed by a different substance showing maximal absorption near 275 $\mu\mu$.

Concentration of a '270 $\mu\mu$. substance'. At the time this work was done it was assumed that the absorption peak near 270 $\mu\mu$. was due to a single substance and that as this was concentrated the intensity of absorption would rise to a maximum. It now seems as if this assumption led to the mixing of fractions which might better have been kept apart.

Various fractions obtained from the chromatography of liver unsaponifiable matter from the first and second horses were combined (0.192 g.) and again chromatographed twice (on alumina plus 5%, v/w, water) to yield 0.048 g. of material showing $E_{1\text{cm}}^{1\%}$ 272 $\mu\mu$., 206. This was combined with another preparation (0.034 g. $E_{1\text{cm}}^{1\%}$ 272 $\mu\mu$., 209) and gave on chromatography a fraction $\lambda_{\max}$ 269 $\mu\mu$., $E_{1\text{cm}}^{1\%}$ 242 (0.049 g.), together with a small fraction showing $\lambda_{\max}$ 274 $\mu\mu$. and an inflexion at 330 $\mu\mu$. The main fraction gave no colour with the SbCl_3 reagent and contained no nitrogen.

Third horse liver. This came from an aged horse and contained but little vitamin A. From 3.75 kg., the final yield of the '270 $\mu\mu$. substance' was 0.036 g. $E_{1\text{cm}}^{1\%}$ 174.

This was added to the best previous product ($E_{1\text{cm}}^{1\%}$ 242) and the whole rechromatographed on watered (5%, v/w) alumina to give on elution with 10% (v/v) ether–light petroleum 0.031 g. of material with $\lambda_{\max}$ 270 $\mu\mu$. (half width 34 $\mu\mu$.) $E_{1\text{cm}}^{1\%}$ 352 with a very flat portion from 300–400 $\mu\mu$., $E_{1\text{cm}}^{1\%}$ 10–15 and 0.026 g. of less intensely absorbing material, qualitatively similar. At this stage the $E_{1\text{cm}}^{1\%}$ 352 material was an orange-yellow resin. (Found: C, 82.94, 82.88; H, 11.29, 11.27; O (by difference), 5.77, 5.85%. Mol.wt. (Rast) 430. It seemed highly probable that the material was a mixture of two similarly absorbing substances but having different intensities of absorption. A mixture of equal parts of $\text{C}_{27}\text{H}_{44}\text{O}_2$ and $\text{C}_{27}\text{H}_{42}\text{O}$ would give C, 82.8; H, 11.1; O, 6.1%; mol.wt. 391.)

Fourth horse liver. This tissue was obtained from a healthy horse slaughtered for meat at the abattoir. The vitamin A content was high, about 500 i.u./g.

From the earlier work it seemed clear that substantial amounts of horse liver unsaponifiable matter would be needed. Experiments were accordingly carried out to see if liver could be cooked and dried so as to yield its fat readily to a solvent in a small industrial plant. The fat could then be saponified conveniently.

The liver (2–3 kg.) was cut up into slices (about 200 g.) which were suspended on hooks in an 8 gal. pressure cooker containing 200 ml. water, and cooked at 40 lb./sq.in. pressure (40 min.). The liver, now friable, was removed, the larger ducts were separated and the bulk of the tissue was minced and then dried on trays at 92°. The dried liver was extracted with trichloroethylene in a pilot plant by Messrs J. Bibby and Sons, to whom we are greatly indebted.

In one run 2.765 kg. of liver gave 0.775 kg. dried tissue, which yielded 63 g. fat and in turn 6 g. of unsaponifiable matter. The yield of '270 $\mu\mu$. substance' was encouraging.

First large batch of horse liver. Accordingly, 43 kg. of liver were treated in the above manner and gave 2.17 kg. of crude lipid. The vitamin A content of about 150 i.u./g. liver

was lower than was expected although still high. The unsaponifiable fraction (122 g.) showed a fairly typical vitamin A spectrum with irrelevant absorption below 325 m μ . Cholesterol (51 g.) was crystallized from methanol and removed. The 'cholesterol-free' unsaponifiable matter was chromatographed on a large column of alumina (weakened by water, 5%, v/w). Excellent vitamin A fractions were obtained, although saponification did not appear to have been complete. Rechromatography gave only two small fractions in which the 270 m μ . peak alone was shown, and vitamin A artifacts were formed in the larger fractions with continued manipulation. Many fractions with $\lambda_{\max}$. 270–273 m μ . accompanied by material showing broad bands with peaks at 335–340 m μ . were obtained, and some fractions contained retinene₁ (vitamin A aldehyde) with $\lambda_{\max}$. 373 m μ . (664 m μ . with SbCl₃).

The duct tissue which, as mentioned earlier, had been separated, was dried, then minced and extracted (ether). The lipid showed $\lambda_{\max}$. 270 m μ ., $E_{1\text{ cm.}}^{1\%}$. 6–16 in different experiments. Unsaponifiable matter (2.9 g.) was crystallized from methanol and the cholesterol removed. Chromatography of the residue on alumina (weakened by water, 5%, v/w) gave a fraction with $\lambda_{\max}$. 270 m μ ., $E_{1\text{ cm.}}^{1\%}$. 108. This on re-adsorption on similar alumina gave two fractions eluted by 2% ether–light petroleum (a) 15 mg. $E_{1\text{ cm.}}^{1\%}$. 269 m μ ., 178 and (b) 50 mg. $E_{1\text{ cm.}}^{1\%}$. 269 m μ . 147; both had weak inflexions near 330 m μ .

From this and in view of the small yield of 270 m μ . material from the unsaponifiable material of the lipid from the duct-free liver, it seemed possible that a '270 m μ . substance' ($E_{1\text{ cm.}}^{1\%}$. 200) was preferentially associated with the duct tissue.

Further large batches of horse liver were obtained from healthy horses slaughtered for meat. As the experiment of drying liver (followed by extraction of lipid and later of unsaponifiable matter) had failed to yield as much 270 m μ . substance as had been hoped, 2 kg. portions of tissue were subjected to alkali digestion. Systematic chromatography after crystallizing cholesterol again resulted in a number of interesting fractions, but as the successive separations proceeded, more trouble from artifacts was met with. The results will not be described in detail; some of the fractions obtained were used later.

Concentration of the '270 m μ . substance'. One fraction (0.45 g.) containing a little of the '270 m μ . substance' was crystallized from light petroleum without effecting any great separation: crystals $\lambda_{\max}$. 271 m μ ., $E_{1\text{ cm.}}^{1\%}$. 21, non-crystalline matter $\lambda_{\max}$. 268 m μ ., $E_{1\text{ cm.}}^{1\%}$. 31. Fractions similar to these were bulked but rechromatography did not raise the $E_{1\text{ cm.}}^{1\%}$. value beyond about 280, and such fractions were small (2–3 mg.).

Five other fractions ($E_{1\text{ cm.}}^{1\%}$. 95–237 at $\lambda_{\max}$. 269–270 m μ .) were bulked (0.178 g.) and rechromatographed on 5% (v/w) watered alumina. The most intensely absorbing fraction (27 mg.), a light petroleum eluate with $\lambda_{\max}$. 269 m μ ., $E_{1\text{ cm.}}^{1\%}$. 534 was crystallized at low temperature from light petroleum to raise the $E_{1\text{ cm.}}^{1\%}$. to 557 at 268 m μ . with hardly any absorption on the long-wave side of 300 m μ .; the crystals, m.p. 108–110° were yellowish white. (Found: C, 77.29; H, 10.52; O (by difference), 12.11%; mol.wt. (Rast) 319. C₂₇H₄₂O₃ requires C, 78.2; H, 10.2; O, 11.6%; mol.wt. 414. C₂₇H₄₄O₃ requires C, 77.8; H, 10.7; O, 11.5%; mol.wt. 416.) The material had no vitamin D activity (we are indebted to Mr F. H. Moul of Lever Bros. Ltd., Cheshire for this test), nor did it give a colour with either the SbCl₃ or Liebermann–Burchard reagents.

Further batches of liver yielded 189.2 g. of unsaponifiable matter from which 89.3 g. of cholesterol was removed. Attempted separations by partition methods or formation of ketone derivatives met with little success.

The results of chromatography on the 'cholesterol-free' unsaponifiable matter are not reported in full, but among the fractions obtained were:

(a) '284 m μ . substance': $\lambda_{\max}$. 284 m μ ., $E_{1\text{ cm.}}^{1\%}$. 326 ($\lambda_{\max}$. 296 in chloroform). This material which was only weakly adsorbed on watered (5%, v/w) alumina, was not obtained pure.

(b) A white crystalline material with practically no selective absorption and a very sharp m.p. 252–252.5° was isolated while trying to crystallize the above '284 m μ . material' from light petroleum at about 0°. It was probably a steroid.

(c) White waxy crystalline material from the most weakly adsorbed fractions (m.p. 56.2–57.7° $\lambda_{\max}$. 260 m μ ., $E_{1\text{ cm.}}^{1\%}$. 0.7 very weak), iodine val. 3.5. The infrared adsorption spectrum (film) [peaks at 4300, 4100, 2900, 2806, 1460, 1380, 1124 (963 and 910 very weak), 886, 731, 721 cm.⁻¹] agreed with that of a paraffin hydrocarbon slightly contaminated with unsaturated material (which fluoresced blue in ultraviolet light). From its m.p. the hydrocarbon was perhaps hexacosane (C₂₆H₅₄), but hydrocarbons C_nH_{2n+2}, where $n = 26$ –34 differ little in percentage composition and identification is difficult:

Found: C, 84.9, 85.03; H, 14.36, 14.64. Mol.wt. 482 (Rast)

C ₂₆ H ₅₄	85.13	14.86	366
C ₂₇ H ₅₆	85.15	14.84	370
C ₂₈ H ₅₈	85.17	14.82	394
C ₃₄ H ₇₀	85.25	14.75	478

(d) A liquid fraction (fluorescing purplish blue in ultraviolet light) with low intensity ultraviolet absorption and iodine value 203 was also obtained from the weakly adsorbed fractions mentioned in (c) above. On treatment with dry HCl this gave a crystalline solid shrinking at 100–105°, softening at 108–110°, melting 112–115°. This could have been squalene hexahydrochloride (m.p. indefinite 107–115°, Heilbron, Kamm & Owens, 1926) or heptene octahydrochloride (m.p. 115–121°, Channon, 1926):

	C (%)	H (%)	Cl (%)
Squalene hydrochloride	57.2	9.0	33.9
Heptene hydrochloride	59.5	9.3	31.2
Found	60.6	9.3 ₅	31.5

The analytical results point to heptene and not squalene but one small preparation of white hydrochloride crystals contained 34.8% Cl and melted at 110°.

(e) A small amount of waxy crystals, m.p. 84°, was obtained, ultraviolet absorption negligible. (Found: C, 74.98; H, 13.09. C₆H₁₀O requires C, 74.4; H, 13.2%.)

Isolation of a '270 m μ . substance'. A large number of early eluates (i.e. weakly adsorbed fractions) were combined and rechromatographed on full strength alumina. From 11 g. after two adsorptions 4.5 g. of material showing $\lambda_{\max}$. 269 m μ ., $E_{1\text{ cm.}}^{1\%}$. 60 (in 60–80° light petroleum) but contaminated with carotene was obtained in the petrol eluates. From this, after further rechromatography on similar alumina, two fractions were eluted with 10 and 20% (v/v) ether–light petroleum with $E_{1\text{ cm.}}^{1\%}$. values 100 and 268 respectively. These were separately rechromatographed (see Tables 2 and 3).

Table 2. *Rechromatography of 0.36 g. of a horse liver fraction with $E_1^{1\%}$ 268 at 266 m μ .*

20 g. alumina; 10 $\times$ 1.7 cm. Absorption spectra in purified petroleum b.p. 60–80°. P=Light petroleum; E=diethyl ether.

Fraction	Eluent	Vol. (ml.)	Wt. (g.)	$\lambda_{\max.}$ (m μ .)	$E_1^{1\%}$ cm.
1	P	800	0.002	255–280	37
2	P	500	0.113	267 (273 and 330)	415
3	P	250	0.029	267	542
4	P	250	0.033	267	531
5	10% E in P	250	0.017	267	301

(Other fractions not reported.)

Table 3. *Rechromatography of 0.3 g. of horse liver fraction $E_1^{1\%}$ 100 at 267 m μ .*

20 g. alumina 10 $\times$ 1.7 cm. Absorption spectra in purified petroleum b.p. 60–80°. P=Light petroleum; E=ether.

Fraction	Eluent	Vol. (ml.)	Wt. (g.)	$\lambda_{\max.}$ (m μ .)	$E_1^{1\%}$ cm.
3	7.5% E in P	250	0.008	266 275	650
4	10% E in P	500	0.032	266 274	560
5	20% E in P	500	0.027	266–271	83

(Other fractions not reported.)

Table 4. *Fractionation of horse kidney unsaponifiable matter*

537 g. wet wt., 1.72 g. unsaponifiable. Cholesterol removed, 1.3 g.; alumina, 22 g. plus water 5% (v/w). P=Light petroleum; E=ether. Absorption spectra 200–400 m μ . in cyclohexane, and 400–500 m μ . in purified petroleum 60–80°.

Fraction	Eluent	Vol. (ml.)	Wt. (g.)	$\lambda_{\max.}$ (m μ .)	$E_1^{1\%}$ cm.	Remarks
1	P	40	0.02	255	28	Opaque wax
2	P	80	0.0024	266 336 448 474	136 83 466 393	Carotene fraction — — —
3	P	65	Negligible	—	—	Yellow resin
4	5% E in P	30	0.0023	Negligible	—	—
5	5% E in P	70	0.0031	270	136	?SA*
6	10% E in P	50	0.019	274	19	?
7	20% E in P	50	0.018	274	114	?
8	E	50	0.192	275	2.9	White solid

* Festenstein, Heaton, Lowe & Morton (1955).

Table 5. *Fractionation of horse spleen unsaponifiable matter*

627 g. tissue; 2.44 g. unsaponifiable. Cholesterol removed 1.54 g.; alumina 20 g. plus H₂O, 5% (v/w). P=Light petroleum; E=ether. Absorption spectra 200–400 m μ . in cyclohexane, and 400–500 m μ . in purified petroleum b.p. 60–80°.

Fraction	Eluent	Vol. (ml.)	Wt. (g.)	$\lambda_{\max.}$ (m μ .)	$E_1^{1\%}$ cm.	Remarks
1	P	50	0.04	250–260 325	61	Opaque wax
2	P	90	0.004	262–263 336 448 474	133 77 335 277	Carotene fraction
3	P	125	Negligible	255	—	Yellow resin
4	5% E in P	30	0.002	253	—	—
5	5% E in P	70	0.002	268	130	—
6	10% E in P	50	0.017	276	19	?
7	20% E in P	50	0.013	270	—	?
8	E	50	0.166	270 280	—	White solid

A number of good fractions were combined and subjected to repeated crystallization at low temperature; light petroleum is the best solvent but acetone and methanol were tried at first. The final product melted sharply at 111.5–112.5° and consisted of almost white crystals, $\lambda_{\max}$, 267 m μ ., $E_{1\text{cm}}^{1\%}$, 644 in purified 60–80° light petroleum, 269 m μ ., $E_{1\text{cm}}^{1\%}$, 641 in cyclohexane, 277 m μ ., $E_{1\text{cm}}^{1\%}$, 625 in ethanol and 278 m μ ., $E_{1\text{cm}}^{1\%}$, 637 in chloroform. (Found: C, 84.18, 84.29; H, 10.74, 10.68; O (by difference), 5.08, 5.03%. Mol.wt. (Rast) 406.) Authentic cholesta-3:5-dien-7-one. Found: C, 84.65; H, 10.63. Calc. for $\text{C}_{27}\text{H}_{40}\text{O}$: C, 84.74; H, 11.07%. Mol.wt. 382 (see below).

Characterization as cholesta-3:5-dien-7-one. The substance isolated was clearly an unsaturated ketone and was identified as cholesta-3:5-dien-7-one by comparison with authentic material prepared from cholesterol (Daniel, Lederer & Velluz, 1945). The 'synthetic' material, m.p. 113–114°, showed no depression when admixed with the isolated product. Infrared absorption spectra were almost identical and agreed with the results given by Jones & Dobriner (1949). Ultraviolet absorption spectra agreed, both materials showing the shift of $\lambda_{\max}$ from 267 to 277 m μ on changing the solvent from light petroleum (60–80°) to ethanol. Both preparations showed in conc. H_2SO_4 strong selective absorption with $\lambda_{\max}$, 355 m μ . Optical rotations given in the literature indicate $[\alpha]_D$ about -300° in chloroform. Our synthetic sample gave $[\alpha]_D^{25} = -298 \pm 4^\circ$ in chloroform and crystals of the horse liver compound recovered from mother liquors in the course of isolation showed $-261 \pm 5^\circ$ chloroform.

Horse kidney and spleen

Kidney and spleen tissue was obtained from healthy animals slaughtered for meat. After disintegrating in a Waring Blender in the presence of a little ethanol the tissue was warmed with ethanolic KOH for 4 hr. and left overnight. The extraction was made with light petroleum which was finally removed at 30–35° under suction in an all-glass apparatus. The unsaponifiable fractions, which were both orange-yellow, partially crystalline solids, had the following absorption spectra:

Kidney: $\lambda_{\max}$, 268 and 450–455 m μ ., $E_{1\text{cm}}^{1\%}$, 1.6 and 0.9 inflexions at 330, 420, 480 m μ . (SbCl_3 colour pale green-blue, weak band at 620 m μ .).

Spleen: $\lambda_{\max}$, 255–260 and 450–455 m μ ., $E_{1\text{cm}}^{1\%}$, 2.6 and 1.3, inflexions at 330, 425, 475 m μ . (SbCl_3 colour pale green-blue, weak band at 590 m μ .). These materials, after removal of cholesterol, were chromatographed on alumina. The results are shown in Tables 4 and 5.

It is evident that the kidney and spleen of the horse both contain carotene and very small amounts of substances absorbing maximally near 270 m μ . This material is almost certainly the same as the substance SA of Festenstein, Heaton, Lowe & Morton (1955).

DISCUSSION

With small amounts of material, chromatography on alumina effects useful separations of unsaponifiable lipids even in the presence of relatively considerable amounts of vitamin A. On the other hand, the search for minor constituents must involve large amounts of material and prolonged manipula-

tion; as a result, vitamin A artifacts are formed freely. Among the likely products are various epoxides of vitamin A and retinene. Some of these have absorption maxima at 275–285 m μ . On the other hand, the vitamin A of herbivora owes its origin to fission of β -carotene and the process need not of necessity be restricted to the central (15–15') double bond; it is surprising that the alcohols $\text{C}_{15}\text{H}_{23}\text{OH}$ (with three double bonds) and $\text{C}_{25}\text{H}_{35}\text{OH}$ (with seven double bonds) have not been found. Such compounds might yield the corresponding aldehydes and the C_{15} aldehyde might be chromophorically akin to a vitamin A epoxide.

The present investigations on liver oils point to the existence of more than one constituent with $\lambda_{\max}$, 269–272 m μ ., which fail to give colour tests with the SbCl_3 reagent. The substances could be true natural products or artifacts; one is a steroid (cholesta-3:5-dien-7-one) but the others could be either sterol derivatives or congeners of vitamin A derived from dietary carotene by fission followed by reduction or epoxidation. The problem of their nature will be discussed in later papers, but some aspects of the present work can be usefully discussed at this stage (see also Festenstein *et al.* 1955).

Fluorescent material from ox liver

Florsheim & Krichevsky (1950) obtained from liver unsaponifiable matter (bulked extracts from livers of cancer patients and of experimental rabbits) a fraction with maxima at 272 and 308 m μ with an inflexion near 245 m μ . The material exhibited intense blue fluorescence under ultraviolet illumination.

Morice (1951) obtained 40 mg. of white crystals from 4 kg. of New Zealand butter. The properties of the substance, m.p. 108°, mol.wt. 232 (Rast) and analysis indicated $\text{C}_{18}\text{H}_{16}\text{O}$ (mol.wt. 260). Our material from ox liver lipid is quite evidently the same compound; the chance that a spectrum so complicated as that recorded in Fig. 1 should be qualitatively and quantitatively reproduced by two different substances is practically negligible. It seems very likely that the same compound was present in Florsheim & Krichevsky's fractions. Morice obtained negative results in trying to prepare an oxime or an ester. In our hands the ultraviolet absorption and fluorescence persisted unchanged (a) in ethanolic potassium hydroxide, (b) in acidified ethanol, (c) after exposure to nascent hydrogen (sodium dissolving in ethanol), (d) after treatment in ether with lithium aluminium hydride, (e) after treatment with permanganate in acetone. The fluorescence was well seen in ethanol, acetone, light petroleum and cyclohexane.

Florsheim & Krichevsky (1950), however, did not observe fluorescence in ethereal hydrogen chloride but recorded its reappearance on neutralization.

The characteristic ultraviolet absorption was changed to an inflexion at 255–265 $m\mu$. on treatment with potassium permanganate in acetone, but blue fluorescence persisted. We have obtained highly fluorescent hydrocarbon fractions from horse liver, the only selective absorption being at 258 $m\mu$. It is impossible in these cases to be sure that the selectively absorbing material is the same as that which gives rise to the fluorescence.

From the absorption spectrum and from the formula $C_{19}H_{16}O$ of Morice (1951) the substance responsible must be polycyclic and highly unsaturated. The spectrum has something in common with both 1- and 2-naphthalacetone as well as 2-naphthol and the formula would fit a substituted chrysene or a compound with a cyclopentenophenanthrene ring system, eight double bonds and a CO or OH group, with either one ethyl or two methyl groups as substituents. The compound has not been identified and we have failed to find in the literature any absorption curve for a pure compound which would establish the chromophoric groupings (cf. Friedel & Orchin, 1951). The substance has recently been again obtained in minute amounts by Dr J. S. Lowe and Mr F. W. Heaton in this laboratory. It was traced to a sample of alumina used for chromatography. The amount present (about 0.2 mg./100 g.) is small and its origin is not known. Other samples of alumina, after weakening with water, yielded to ether very small amounts of organic material with inflexions at 233 and 260 $m\mu$. The extracted material was brilliantly blue fluorescent in ultraviolet light.

The substances are thus laboratory contaminants rather than normal constituents of animal lipids.

Cholesta-3:5-dien-7-one

This compound has been isolated on numerous previous occasions from natural products. Ruzicka & Prelog (1943) obtained it from pig testes; Hardegger, Ruzicka & Tagmann (1943) got it from arteriosclerotic aortas; Prelog, Ruzicka & Stein (1943) obtained it from pig spleen; Daniel *et al.* (1945) from wool fat of sheep and Karrer & Naik (1948) obtained it from ox liver.

Blix & Lowenhielm (1928) found that cholesterol in colloidal aqueous solution was readily attacked by molecular oxygen at room temperature to give 'oxycholesterol'. Alkali soaps catalysed the oxidation. Bergström & Wintersteiner (1941; 1942*a, b*) stabilized the aqueous dispersion of cholesterol with sodium stearate and aerated at 85°. After several hours the reaction came to a standstill. The primary point of attack was C-7 to give 7-oxocholesterol and 7 α - and 7 β -hydroxycholesterol.

These autoxidation experiments indicate that when any 7-oxygenated sterol is isolated from a

natural product the possibility that it is an artifact must be considered.

It is, however, likely that the cholesta-3:5-dien-7-one isolated from wool fat is an artifact, because when Daniel *et al.* (1945) saponified with sodium methoxide in anhydrous ether in the cold (rather than by boiling with hot ethanolic potassium hydroxide) the product did not exhibit strong negative optical rotation. This result cannot, however, be safely given general application, because wool fat may well have been oxidized by air as far as the dienone precursor (7-oxocholesteryl ester) before saponification. Such oxidation need not occur in all lipid material; thus it is significant that brain tissue which is very rich in cholesterol did not produce the dienone even after saponification in hot ethanolic potash and working up in the ordinary way (Kantiengar & Morton, 1955). For the present it must be concluded that although the cholesta-3:5-dien-7-one may be an artifact, it is not safe to assume that it is necessarily so.

Unidentified substance with $\lambda_{\max}$ near 270 $m\mu$.

The material with $\lambda_{\max}$ 272 $m\mu$. and designated SA, which was isolated from horse intestine and other sources by Festenstein, *et al.* (1955) is distinct from cholesta-3:5-dien-7-one. It can easily be confused with that substance because of the very close resemblance in ultraviolet absorption and chromatographic properties. The two substances differ, however, in respect of their absorption spectra in conc. H_2SO_4 ($\lambda_{\max}$ 315 $m\mu$. for the '272 $m\mu$. substance', 355 $m\mu$. for the dienone). The cholesta-3:5-dien-7-one, moreover, seems to be more stable both to light and to oxygen.

It seems very likely that in much of the work recorded in the present paper, the 272 $m\mu$. substance and the dienone were often obtained together. Even this, however, does not fully account for the analytical findings and the molecular weight of the material of m.p. 108–110° (p. 278). Although this is similar to cholesta-3:5-dien-7-one in respect of its spectrum, it contains much more oxygen, probably three atoms in the molecule. The same chromophoric unit could be present in both molecules. It will be shown in later papers that fractions indistinguishable from the '272 $m\mu$. substance' have been obtained from normal rat liver and from normal cockerel liver.

Other compounds

Cholest-4-ene-3 β :6 β -diol (m.p. 254°) has been isolated from pig spleen (Prelog *et al.* 1943) and from photooxidized cholesterol (Windaus, Bursian & Riemann, 1941). Our material of m.p. 252–252.5° may have been this substance but there was too little for full investigation. Haslewood (1941) isolated cholestane-3 β :5 α :6 β -triol (the so-called

'trans-triol') from ox liver. He also obtained an alcohol m.p. 93–95° with a possible formula $C_{24}H_{46}O$. The transparent material m.p. 84° isolated from horse liver and analysing for $(C_8H_{17-18}O)_n$ is a compound of possibly similar type.

Hydrocarbons

The occurrence of saturated hydrocarbons in mammalian liver has been reported by Channon, Devine & Loach (1934), Bürger & Plötner (1940), Dimter (1941), Stanger, Steiner & Bolyard (1944). Prelog *et al.* (1943) isolated a hydrocarbon of m.p. 54° from pig spleen. Heptacosane ($C_{27}H_{56}$, m.p. 59°) and probably pentacosane ($C_{25}H_{52}$, m.p. 52–54°) are reported by Hart & Northrup (1935) to occur in human pregnancy urine. Marker (1938) obtained octacosane ($C_{28}H_{58}$, m.p. 63°) from stallion urine and cow's pregnancy urine. Prelog, Führer, Hagenbach & Frick (1947) obtained *n*-nonacosane ($C_{29}H_{60}$) from pregnant mare's urine. MacKenna, Wheatley & Wormall (1952) and Festenstein & Morton (1952) have found a hydrocarbon m.p. 57.6° in human sebum. The compound isolated from horse liver m.p. 57° was clearly similar, but the exact number of carbon atoms has not been established.

Hepene ($C_{45}H_{76}$) and squalene ($C_{30}H_{50}$) are the only well-characterized open-chain unsaturated hydrocarbons found in animal tissues. Squalene has recently been found in sebum (MacKenna *et al.* 1952; Festenstein & Morton, 1952). It has been suggested (Prelog *et al.* 1943) that hydrocarbons isolated from animal tissues may have been introduced from outside during manipulation, e.g. in lubricants, but this seems an unlikely explanation for either hepene or squalene.

Carotenoids and vitamin A derivatives

Carotenoids were present in all the liver lipids examined except the shark and fin-whale liver oils. The sheep-liver oil contained less than ox or horse-liver oils. In addition to β -carotene, the sheep and ox chromatograms gave indications of lycopene but the horse liver 'carotene' was apparently a single substance. Little xanthophyll material is stored in horse liver.

Although the livers from horses killed by the knacker were not devoid of carotenoids, they contained very little. The vitamin A content was also very low and presumably the horses had not had access to pasture for a long time.

Jensen & With (1939) found about 500 i.u./g. vitamin A in horse liver, while Rudra (1946) recorded a range of 32–2000 i.u./g. (mean 628 i.u./g.). Wanntorp (1947) observed an average of 400 i.u./g. for animals killed in the summer after summer grazing. Our data for healthy animals slaughtered for meat (91–500 i.u./g.) are consistent

with the above, but the figures for the worn-out horses 5–23 i.u./g. are very low indeed. Nevertheless, it is practically certain that even these animals had not been depleted of vitamin to the point of metabolic abnormality (see Lowe, Morton & Harrison (1953) associated with exhaustion of vitamin A reserves).

The fact that it was easier to obtain the fractions showing a single absorption band with $\lambda_{\max}$ near 270 m μ . from the livers low in vitamin A than from those high in vitamin A might have been the result of reduced interference from vitamin A and its derivatives. It is not easy to prove the contrary but the possibility of a real increase of such substances in vitamin A depletion is under study.

The evidence concerning the nature of the vitamin A artifacts needs to be summarized, although it is not conclusive. Retinene₁ (vitamin A aldehyde), $\lambda_{\max}$ 373 m μ . in cyclohexane 664 m μ ., $SbCl_3$ colour test, was encountered occasionally. A related substance (Meunier & Jouanneteau, 1948) said to have the structure (I) has $\lambda_{\max}$ 345 m μ . in chloroform and 560 m μ . in the $SbCl_3$ colour test. This was not encountered, nor was another compound (Meunier, Zwingelstein, Jouanneteau & Mallein, 1950) with maxima at 255, 290 and 340 m μ ., $SbCl_3$ colour test 545 and 490 m μ ., the suggested structure of which is (II).

Neither these compounds nor the known vitamin A epoxides would suffice to explain all the absorption curves recorded, and it still remains possible that liver oils contain small amounts of unidentified vitamin A congeners. This problem is being approached from a different angle.

The questions raised by the present work are whether vitamin A status influences sterol metabolism and whether cholestadienone, the '272 m μ . substance' SA, or the hydrocarbons are (upward or downward) on sterol metabolic pathways. These problems will be discussed later.

SUMMARY

1. Liver fat and unsaponifiable fractions therefrom have been subjected to extensive chromatography with spectrophotometric control. Irre-

spective of the source (Argentine shark, fine-whale, sheep, ox, horse) formation of a variety of vitamin A artifacts handicaps the search for new minor constituents.

2. One sample of ox liver fat unsaponifiable matter yielded a compound with a highly characteristic absorption spectrum quantitatively indistinguishable from that of a compound $C_{16}H_{18}O$ isolated by Morice from butter fat. Its origin is unknown, but it is much more likely to be a casual contaminant than a normal lipid constituent.

3. A lipid unsaponifiable constituent with $\lambda_{\max}$ 270 $m\mu$. has been isolated from horse liver and identified with cholesta-3:5-dien-7-one. The question whether it is or is not an artifact remains open but the balance of evidence is in favour of its occurrence as such. A second substance with a very similar absorption spectrum ($\lambda_{\max}$ 272 $m\mu$.) has been obtained; it is richer in oxygen than the dienone and probably contains three oxygen atoms in the molecule.

4. A saturated higher aliphatic hydrocarbon and an unsaturated hydrocarbon, possibly heptene ($C_{26}H_{54}$) have been isolated from horse liver.

5. Carotenoids (mainly β -carotene) are present in horse and ox liver and to a lesser extent in sheep liver.

6. The unsaponifiable fractions from horse kidney and spleen lipids contain in addition to cholesterol a waxy fraction (probably hydrocarbon), β -carotene, small amounts of a substance with $\lambda_{\max}$ about 270 $m\mu$. and of another substance with $\lambda_{\max}$ 274–276 $m\mu$.

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