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Studies in Vitamin A

19. PREPARATION AND PROPERTIES OF RETINENE$_2$

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(*Received* 9 *February* 1952)

Retinene$_2$ was first recognized by Wald (1937) and described as a 'deep yellow carotenoid' obtained from the retinas of freshwater fishes; when dissolved in chloroform it showed a wide absorption band with $\lambda_{max.}$ at 405 mμ., and in the presence of excess of the antimony trichloride reagent it gave a greenish blue colour with $\lambda_{max.}$ at 702–706 mμ.

The retinas of freshwater fishes are violet in colour (Kühne & Sewall, 1880), whereas those of most animals and sea fishes are pink. The spectral shift (Köttgen & Abelsdorff, 1896) is now ascribed (Wald, 1937, 1938–9*a*, *b*) to the partial or complete replacement of visual purple (rhodopsin) by porphyropsin with an absorption peak near 522 mμ. instead of 500 mμ.

Exposure to light liberates retinene$_1$ from rhodopsin and retinene$_2$ from porphyropsin, but the amounts obtainable from retinas are of course too small for other than spectroscopic identification. Retinene$_1$ has been shown to be vitamin A$_1$ aldehyde and can now be obtained in quantity by oxidation of vitamin A$_1$ alcohol (Ball, Goodwin & Morton, 1948). Similarly, retinene$_2$ is the aldehyde of vitamin A$_2$ (Morton, Salah & Stubbs, 1947), but its preparation in a state of purity has been difficult.

The term vitamin A$_2$ was first used by Edisbury, Morton & Simpkins (1937) on the strength of the very close analogy between the distribution of vitamin A$_1$ in the organs of marine fishes and the distribution of a '693 mμ. chromogen' in freshwater fishes. The antimony trichloride colour test applied to vitamin A$_1$ gives a blue solution ($\lambda_{max.}$ 620 mμ.), whereas when applied to vitamin A$_2$ it gives a greenish blue solution ($\lambda_{max.}$ 693 mμ.). The ultraviolet absorption peaks are at 325–328 mμ. for vitamin A$_1$ and 350–352, 286 mμ. for vitamin A$_2$. The 693 mμ. chromogen had been described earlier (Heilbron, Gillam & Morton, 1931). Its importance was clearly recognized by Lederer & Rosanova (1937) and Wald (1938–9*a*).

The problems to be discussed in this and succeeding papers have a rather complicated background which needs explanation.

(1) Fish-liver oils with a high vitamin A$_2$ content are not easy to obtain; most commercial oils are of marine origin and vitamin A$_2$, although frequently present, rarely accounts for more than 5–10% of the vitamin A content. For the British freshwater fishes which have been studied, the absolute amount of vitamins A present in liver oils is quite low and the

proportion as vitamin A_2 is approximately one-third to one-quarter of the total.

(2) There is at present no satisfactory method of effecting a clean separation of vitamin A_2 from vitamin A_1 and the best method of preparing retinene$_2$ is first to obtain a sterol-free concentrate of mixed vitamins A_1 and A_2 and then to oxidize the alcohols to a mixture of retinene$_1$ and retinene$_2$. These can then, with difficulty, be separated by chromatography. When the separation is practically complete, further chromatography removes artifacts, and low-temperature crystallization can be used to complete the purification. The retinene$_2$ can then be reduced to vitamin A_2 by means of lithium aluminium hydride.

(3) Retinene$_2$ and vitamin A_2 are very labile substances, particularly unstable when nearly pure and not easy to analyse for carbon and hydrogen. This is a matter of some importance because, as will be shown, much depends on whether the formula of retinene$_2$ is $C_{20}H_{28}O$ or $C_{20}H_{26}O$. Throughout the 6 years over which the present studies have been spread there has been uncertainty about the structural formula of vitamin A_2. The evidence has been so conflicting (see p. 539) that an approach via the structure of retinene$_2$ might be decisive. The dehydrovitamin A_1 formula for vitamin A_2 favoured by Morton *et al.* (1947) has recently been proved by synthesis (Farrar, Hamlet, Henbest & Jones, 1951) and the specification for retinene$_2$ (communicated to them) confirmed, because the synthetic retinene$_2$ obtained by the manganese dioxide oxidation (Ball *et al.* 1948) of synthetic vitamin A_2 agreed with natural retinene$_2$ in all respects.

(4) There are many possibilities of isomerism in vitamins A_1 and A_2 and therefore in the retinenes. If a mixture of neovitamins A_1 and A_2 and *all-trans* vitamins A_1 and A_2 is oxidized there may be four or more retinenes. Each of these is subject to the risk of epoxidation and the formation of other artifacts. Attempts to dehydrogenate vitamin A_1 to form retinene$_1$ and retinene$_2$ by the Oppenauer reaction give rise to a variety of products depending on experimental conditions.

For these various reasons much of the work has been repeated many times. The main results had been obtained 2½ years ago, but much labour has since been devoted to obtaining larger amounts of purified materials for analysis and complete characterization.

EXPERIMENTAL

Materials and methods

Sources of vitamin A_2. (*a*) Pike-liver oil. The livers of pike (*Esox lucius*) obtained from Lake Windermere (by courtesy of the Director, Freshwater Biological Research Station) were extracted by standard methods. The resulting oil was low in vitamins A, but A_2 predominated over A_1 ($E^{1\%}_{1\,cm.}$ 693 mμ. 0·52; 620 mμ. 0·18). The pike is by far the most convenient British freshwater fish to use as a source of vitamin A_2 although the yield is poor. (*b*) Ling-cod liver oil. Of all the marine fish-liver oils so far examined for A_2 that of ling cod (*Ophiodon elongatus*) is much the best. The total vitamin A content is high (sometimes 15%), but there is 7 to 10 times as much A_1 as A_2. Supplies of oil were obtained from the U.S.A. through the good offices of the Ministry of Food and of Parke Davis and Co. (*c*) Lake Nyasa oil. This oil from the livers of large fishes (species uncertain) is the best source known to us of vitamin A_2 ($E^{1\%}_{1\,cm.}$ 693 mμ., 174; 620 mμ., 530; A_2/A_1 approx. 1/3). We are indebted to Marine Oil Refiners of South Africa for help in obtaining the oil.

Manganese dioxide. Two kinds were used: (*a*) a fine grade of commercial laboratory reagent and (*b*) material obtained by mixing equivalent amounts of $MnSO_4$ (cryst.) and $KMnO_4$ in aqueous solution, filtering and washing free from SO_4^{2-} and drying on a porous plate in a desiccator. (Details of an improved preparation are given by Attenburrow *et al.* 1952.)

Light petroleum. In the main the commercial product (b.p. 40–60°) was used, but for spectrophotometry a material purified by stirring with H_2SO_4 (conc.), washing and distilling (b.p. 60–80°) was preferred.

Diethyl ether. In order to eliminate peroxides, ether was redistilled over reduced Fe before use.

Alumina for adsorption. Grade O alumina (Peter Spence and Co.) was weakened by incorporating known amounts of water, 5–10%, which were stirred in slowly under light petroleum. (Percentages of water added are w/w.)

Absorption spectra. These were determined using photoelectric instruments, except that routine colour-test determinations were made on a Hilger-Nutting visual spectrophotometer.

Preparation of retinene$_2$ from ling-cod liver oil

Preliminary. The oil used contained 8 times as much A_1 as A_2. Chromatography on the oil gave a fraction in which the ratio A_2/A_1 was raised to 1/3 but it contained only 5% of the vitamin used. Chromatography on the unsaponifiable fraction gave a better yield of enriched product, but the vitamin A_2 proved unstable, presumably because of the removal of natural antioxidants.

The sterol-free unsaponifiable fraction from the original oil was therefore oxidized over MnO_2 according to Ball *et al.* (1948). A portion of the solution of mixed retinenes was chromatographed on full strength alumina, but gave rise to red artifacts in undesirable quantities. Mixtures of various retinene$_1$ and retinene$_2$ derivatives (obtained with aldehyde reagents) could not be separated easily. Chromatography of oxidized unsaponifiable material on bone meal, magnesia or lime was ineffective or detrimental, but 10% watered alumina permitted appreciable separation.

Preparation of vitamin A_1 and A_2 concentrate. Unsaponifiable fractions were obtained from 100 g. portions of ling-cod liver oil and dissolved in methanol. A first crop of sterol separated overnight at 0°. The filtrate was cooled to −70° and a further crop of crystals removed by low-temperature filtration through a cooled, jacketed, Büchner funnel. The filtrate was diluted with one-third its volume of water and extracted with light petroleum (3 × 50 ml.). The solvent was removed and the residue dried by blowing with N_2 after small additions of ethanol. (Yield 8·8 g., $E^{1\%}_{1\,cm.}$ 617 mμ., 3300; 693 mμ., 500; A_2/A_1 approx. 1/6·5.)

Initial isolation of a 740 mμ. chromogen. The concentrate was dissolved in light petroleum (100 ml./g.) and the solution left to stand over MnO_2 (4 g./100 ml.) in the dark for 4 days with occasional shaking. The solution was then filtered and reduced to small bulk. The oxidized material (4 g.) was chromatographed on a column (20 × 2·5 cm.) of 10% watered alumina. Continuous development with light petroleum gave three ill defined zones; the column was extruded, sectioned, and eluted with $CHCl_3$. The middle zone was the richest in a material giving $\lambda_{max.}$ 740 mμ. in the colour test, and the retinene$_1$ band at 664 mμ. was shown at about one-quarter the intensity found before chromatography. The $CHCl_3$ was removed completely and replaced by light petroleum. The solution was chromatographed as before and the middle zone retained. Two maxima were seen in the colour test, the 664 mμ. peak being twice as intense as that at 740 mμ.

After a third chromatographic separation the middle zone showed the 740 mμ. chromogen predominating over the 664 mμ. chromogen. After a fourth separation a middle fraction showed only the 740 mμ. band ($E^{1\%}_{1\,cm.}$ 3000). After standing, the 740 mμ. colour-test maximum disappeared and a band with $\lambda_{max.}$ 705 mμ. persisted. The fraction when examined in $CHCl_3$ showed $\lambda_{max.}$ 405 mμ. and in *cyclo*hexane 385 mμ. The yield of retinene$_2$ free from retinene$_1$ was only 6 mg., but much retinene$_1$ could be obtained free from retinene$_2$.

Second isolation of retinene$_2$ from ling-cod liver oil. The unsaponifiable matter from 500 g. oil was obtained, partially freed from sterols and oxidized over MnO_2 (100 g.) in light petroleum (1 l.). The filtrate was chromatographed on fifteen columns (25 × 2 cm.) in portions of about 65 ml., on 10% watered alumina. The chromatograms were developed (3 hr.) with light petroleum and the middle portions retained, eluted and combined (yield 7·9 g.; in colour test $E^{1\%}_{1\,cm.}$ 740 mμ. 420; 664 mμ. 2070). This material (approx. 60% retinene$_1$, 10% retinene$_2$) was rechromatographed in two portions. The most strongly adsorbed portion contained 2·5 times as much retinene$_2$ as retinene$_1$. After the next adsorption the most strongly held portion showed approximately equal amounts of the two retinenes and a single absorption peak at 377·5 mμ. A further adsorption gave a fraction showing retinene$_2$ only ($\lambda_{max.}$ 385 mμ. in *cyclo*hexane, 740 mμ. fading to 705 mμ. in the colour test). The best fraction (0·11 g. from half the preparation) showed $E^{1\%}_{1\,cm.}$ 385 mμ. 1300. It was an orange-coloured viscous oil.

Preparation of retinene$_2$ from pike-liver oil

Pike-liver oil (300 g.), accumulated from several batches of pike livers, gave a sterol-free unsaponifiable fraction estimated to contain 75 mg. vitamin A_2. Oxidation over MnO_2 (7 days in darkness) gave a mixture of retinenes which after two adsorptions gave a fraction free from retinene$_1$. A third adsorption gave an upper fraction (4·5 mg.) and a middle fraction (20 mg.) showing $\lambda_{max.}$ 385 mμ. in *cyclo*hexane and 740 mμ. (fading to 705 mμ.) in the colour test. In addition to the main band at 385 mμ. there was an inflexion at 310 mμ.

Preparation of crystalline retinene$_2$

(1) *From ling-cod liver oil.* The unsaponifiable fraction from 800 g. was partially freed from sterols and oxidized as before; after four chromatographic separations 0·36 g. of crude retinene$_2$ free from retinene$_1$ was obtained ($E^{1\%}_{1\,cm.}$ 385 mμ., 900 in *cyclo*hexane). Part (0·24 g.) of this was dissolved in light petroleum (2 ml.) and slowly cooled to −70°. After standing overnight, the supernatant liquor was removed and the sticky deposit (m.p. 57°, $E^{1\%}_{1\,cm.}$ 1320) recrystallized. Recrystallization raised the melting point to 59° and then to 61°; $E^{1\%}_{1\,cm.}$ 385 mμ. 1460; no further rise in melting point was obtained by further recrystallization.

(2) *From ling-cod liver oil.* Several batches of oil were used to prepare crude retinene$_2$ as before, and a fraction showing $E^{1\%}_{1\,cm.}$ 1200 was crystallized from light petroleum (2% w/v). Three recrystallizations gave 30 mg. of retinene$_2$, m.p. 77–78°; solubility about 0·2% in light petroleum. The mother liquors gave a further yield of material of m.p. 77–78°. The analytical results will be discussed later. A third preparation from a new batch of ling-cod liver oil ($A_2/A = 10/1$) was undertaken. After repeated recrystallizations the melting point of retinene$_2$ was unchanged at 77–78° (corr.).

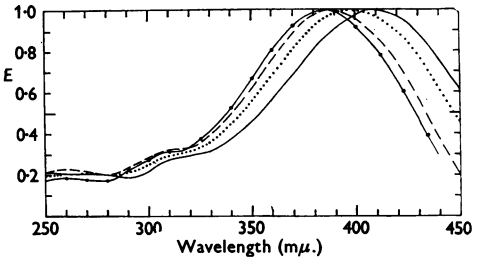


Fig. 1. Absorption spectrum of retinene$_2$ in light petroleum, ●—●; *cyclo*hexane, – – –; chloroform, .——; ethanol, ... (shown as $E_{max.} = 1·0$ to illustrate solvent shifts; for absolute values see Table 1).

Table 1. *Spectroscopic properties of retinene$_2$ (m.p. 77–78°)*

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$	λ of inflexion (mμ.) (approx.)
Light petroleum (40–60°)	385	1490	305
*cyclo*Hexane	386	1440	310
Ethanol	397	1410	315
Chloroform	407	1360	320
*iso*Propanol	396	1440	315
$SbCl_3$ colour test	730–740 Fades to 705	4000*	—

* Initially higher, but after 15–20 sec. is steady between 3800 and 4000.

(3) *From Lake Nyasa oil.* This oil gave 35% of unsaponifiable material which after removal of sterols showed $E^{1\%}_{1\,cm.}$ 326 mμ. ~ 600. The concentrate (30 g. equivalent to 10 g. of mixed vitamins A_1 and A_2) was dissolved in light petroleum (8 l.) and oxidized over MnO_2 (7 g.) for 4 days in darkness with occasional stirring. (The amount of MnO_2 was rather small as it was not desired to convert all the vitamin A_1 to retinene$_1$. Vitamin A_1 preponderates over vitamin A_2 and oxidation causes the 693 mμ. colour-test band of the latter to disappear first; the MnO_2 is then removed by filtration.)

The filtrate was reduced to small bulk, divided into three equal portions which were separately chromatographed on 8% watered alumina (200 g. in each column). Seven zones were seen on development with light petroleum; 2 and 3 (from top) contained most of the retinene$_2$ and 4 and 5 contained increasing amounts of retinene$_1$. The zones were separated by continuous percolation. All fractions enriched in retinene$_2$ were combined and rechromatographed on 10% watered alumina. The whole process was repeated once more and a final chromatography on 11% watered alumina gave 0·75 g. of retinene$_2$ free from retinene$_1$. This material crystallized at low temperature from light petroleum. The melting point (62°) rose with successive recrystallizations (5% solution in light petroleum) to 77–78°. After each crystallization about one-third of the solute is recovered as red crystals about 0·5 mm. long. The spectroscopic properties are shown in Fig. 1 and Table 1.

The preparation of retinene$_2$ is tedious but entirely reproducible given sufficient quantities of a suitable liver oil.

Analysis of retinene$_2$. The pure material is very readily oxidized on exposure to air. This tends to make values for C and H low; the substance is also rather difficult to burn at the ordinary combustion temperatures. Table 2 shows the results of many combustions.

Derivatives of retinene$_2$

(*a*) *Retinene$_2$-2:4-dinitrophenylhydrazone.* An ethanolic solution of 2:4-dinitrophenylhydrazine hydrochloride was added to an equivalent amount of retinene$_2$ in ethanol. On adding 1 drop of conc. HCl and warming gently, an immediate darkening was observed. A good crop of reddish brown needle-like crystals was deposited on cooling in the ice chest. The crystals were centrifuged and recrystallized five times from ethanol containing a little ether.

From retinene$_2$ from ling-cod and pike-liver oils it had m.p. 160–161°, $\lambda_{max.}$ 445 mμ. (EtOH).

From retinene$_2$ from Lake Nyasa oil it had m.p. 188–190°. $\lambda_{max.}$ 460 mμ. $E^{1\%}_{1\,cm.}$ 1240 (acetone), $\lambda_{max.}$ 460 mμ. $E^{1\%}_{1\,cm.}$ 1120 ($CHCl_3$) (see Fig. 2).

(*b*) *Retinene$_2$ semicarbazone.* To an aqueous-ethanolic solution of semicarbazide hydrochloride was added an ethanolic solution of retinene$_2$. The mixture was warmed in a current of inert gas, cooled and extracted with light petroleum-ether mixture (1:1) and the semicarbazone crystallized. It was recrystallized twice from a mixture of light petroleum and ether, when fine yellow crystals were obtained, m.p. 158°, $E^{1\%}_{1\,cm.}$ 1920 at 385 mμ. (EtOH), 1780 at 395 mμ. ($CHCl_3$).

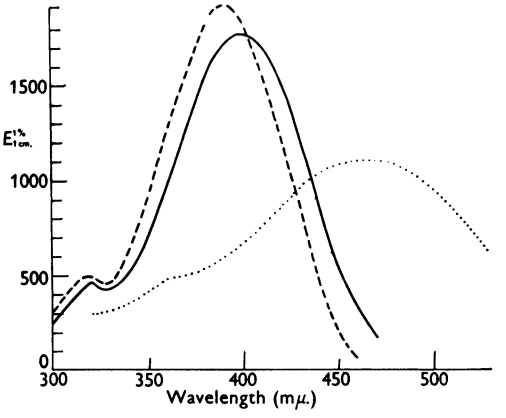


Fig. 2. Absorption spectrum of retinene$_2$-2:4-dinitrophenylhydrazone in chloroform, ·····; retinene$_2$ semicarbazone in ethanol, – – –; and chloroform, ——.

Antimony trichloride colour test

Fig. 3 shows the absorption spectrum of the greenish blue solution measured at definite time intervals after mixing retinene$_2$ (in $CHCl_3$) with the reagent (see Cama, Collins & Morton, 1951). Many solutions were examined at different wavelengths in different order so as to build up the com-

Table 2. *Analytical results on retinene$_2$ (m.p. 77–78°)*

(Analyses by Weiler and Strauss, Oxford, except those marked * which were carried out, with minimal exposure to air, in the Department of Organic Chemistry, University of Liverpool, by courtesy of Prof. A. Robertson, F.R.S.)

	C (%)	H (%)	O (%) (by difference)	Atomic ratio H/C	
Required for $C_{20}H_{26}O$	85·04	9·29	5·66	1·3	
Required for $C_{20}H_{28}O$	84·5	9·86	5·64	1·4	
Retinene$_2$	83·76	9·26	6·98	1·32	
	85·25*	9·28	5·47	1·31	Duplicates
	84·68*	9·34	5·98	1·32	
	83·8	9·0	7·2	1·29	One preparation
	81·14	8·9	9·9	1·31	
	83·5	8·9	7·6	1·28	
	83·36	9·0	7·64	1·29	
	84·0$_2$	9·3	6·67	1·33	One preparation
	84·40	9·7	5·9	1·37	
	84·7	9·6	5·7	1·36	
	84·2	9·5	6·3	1·35	
	85·6*	9·3	5·1	1·30	
	85·3*	9·38	5·32	1·32	
	83·1	8·77	8·13	1·27	
	83·3	8·95	7·75	1·29	

posite curves shown. The maximum slowly drifts from 730 to 740 mμ. as observed visually at the instant of mixing, to 705 mμ. where it remains for a long time.

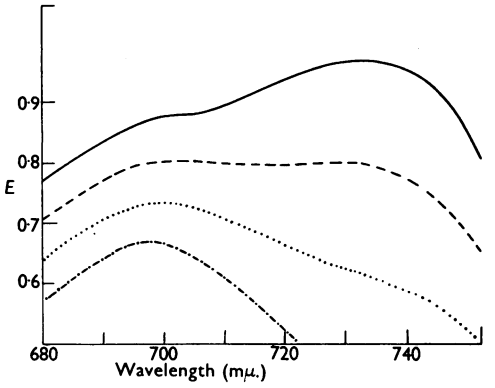


Fig. 3. Absorption curves on blue solution obtained by the interaction of retinene$_2$ and the $SbCl_3$ reagent; illustrating changes with time. ——, each wavelength extrapolated to zero time; – – –, E values at 4 min. after mixing; · · · · ·, E values at 8 min. after mixing; ·–·–·, E values at 12 min. after mixing. (Absolute initial value in Table 1.)

DISCUSSION

If the term retinene$_2$ is restricted to the material obtained by Wald from freshwater-fish retinas, only qualitative descriptive properties are available, namely the ultraviolet absorption spectrum ($\lambda_{max.}$ 405 mμ. in chloroform) and a colour-test maximum at 705 mμ. The fact that our observed colour-test peak is at 730–740 mμ. would preclude identity of the pure material now obtained with Wald's retinene$_2$ were it not for the appearance of the 705 mμ. peak on standing. It is almost certain that Wald missed the initial phase which may, in fact, have been inhibited by congeners.

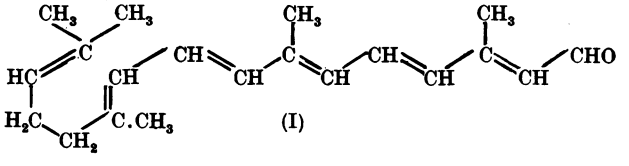
differ from that of retinene$_1$. In fact the curve for retinene$_2$ is appreciably displaced in the direction of longer wavelengths (389 mμ. → 407 mμ. for the peaks in chloroform); this means that retinene$_2$ must have one additional conjugated double bond as compared with retinene$_1$. Further aspects of the structural problem are discussed in the following paper.

The apparent discrepancy over the melting point of the 2:4-dinitrophenylhydrazone has been resolved as follows. Prof. E. R. H. Jones, F.R.S., has informed us that the melting point is 196–197° ($E^{1\%}_{1\,cm.}$ 462 mμ. 1001 in chloroform), but that on heating isomerization occurs to give m.p. 163–167° ($\lambda_{max.}$ 448 mμ. $E^{1\%}_{1\,cm.}$ 866). Our two preparations melted at 188–190° and 160–161° respectively and the spectra agreed closely with those obtained by the Manchester workers.

It is highly probable that natural vitamin A_2 is a mixture of *cis-trans* isomerides and that the crude retinene$_2$ will also be a mixture. This may account for the fact that one preparation showed a melting point of 61° not raised by recrystallization. All later preparations, however, melted sharply at 77–78° after purification, but it is noteworthy that relatively little change in spectrophotometric properties occurred as a result of recrystallization. It may well be that on standing the isomers tend to give an equilibrium mixture and that recrystallization merely accumulates the least soluble isomer.

SUMMARY

1. The liver of ling cod (*Ophiodon elongatus*) contains vitamin A_1 and vitamin A_2 in the ratio 1/8–1/10. The partially sterol-free unsaponifiable fraction in light petroleum left to stand over manganese dioxide gives a mixture of retinene$_1$ and retinene$_2$. Separation can be effected by chromatography on watered alumina.



(I)

It is clear that our retinene$_2$ is an aldehyde derived from the vitamin A_2 of fish-liver oils. It cannot be doubted, in spite of the analytical difficulties revealed in Table 2, that the formula is not $C_{20}H_{28}O$ but $C_{20}H_{26}O$. The different analyses show how oxidation can occur (perhaps with formation of epoxides) or combustion can be incomplete. The structure (I) $C_{20}H_{28}O$ (related to lycopene) is definitely excluded. In any case, the ultraviolet absorption spectrum of such a compound should not

2. The liver oil of pike (*Esox lucius*), though not a very rich source of vitamins A, contains more A_2 than A_1 and the unsaponifiable fraction after oxidation contains the two retinenes, which can be separated.

3. Liver oil obtained from mixed large freshwater fishes of Lake Nyasa contains up to 15 % of mixed vitamins A (A_2/A_1 about 1/3). The sterol-free unsaponifiable fraction yields mixed retinenes on oxidation by manganese dioxide.

4. One preparation of retinene$_2$ showed m.p. 61° (not raised by recrystallization) but numerous subsequent preparations melted sharply at 77–78°. Retinene$_2$ is an aldehyde ($C_{20}H_{26}O$, forming a 2:4-dinitrophenylhydrazone and semicarbazone. Structures requiring $C_{20}H_{28}O$ are excluded. The absorption curves in several solvents are recorded e.g. 386 mμ. for $\lambda_{max.}$ and $E^{1\%}_{1\,cm.}$ 1440 in *cyclo*hexane. The antimony trichloride colour test is greenish blue, $\lambda_{max.}$ 740 mμ. initially, fading to 705 mμ.

5. Retinene$_2$ as obtained above agrees in its properties with the material obtained from bleached freshwater-fish retinas.

We are indebted to the Medical Research Council for financial assistance.

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Studies in Vitamin A

20. SOME PROPERTIES OF RETINENE$_2$

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(*Received* 9 *February* 1952)

In those species which make use of rhodopsin, vitamin A_1 and retinene$_1$ are the main chemical links to the chemistry of scotopic vision and up to the present no alternative to vitamin A_1 has been suggested as the key substance in photopic vision. The reactions of retinene$_1$ with amino compounds have already been described (Ball, Collins, Dalvi & Morton, 1949). The behaviour of retinene$_2$ needs to be compared with that of retinene$_1$ in all respects which seem to have relevance to vision. Special interest attaches to indicator yellow$_2$ observed by Wald (1937, 1938–9*a*, *b*) in bleached freshwater-fish retinas and in Granit's electrophysiologically recorded modulators (Granit, 1947).

EXPERIMENTAL

Reactions of retinene$_2$ with amino compounds

General procedure. (*a*) A solution (4×10^{-5} M) of crystalline retinene$_2$ (Cama *et al.* 1952) in ethanol was prepared ($E_{1\,cm.}$ about 1·5 at 390–400 mμ.). To the solution (2 ml.) was added an aqueous solution (2 ml.) of an amino compound. In some cases the latter solution was nearly saturated but the concentration was always 10^{-3} M. To the mixture was added 0·1 N-NaOH (2 ml.) and the solution was left to stand at room temperature in the dark for 15–30 min. The absorption spectrum was then measured. The solution was then acidified (1 drop conc. HCl) and the absorption spectrum redetermined. For the alkaline solutions $\lambda_{max.}$ occurred at 380–400 mμ. and for the acidified solutions at 460–465 mμ.

(*b*) Amino compounds in which the —NH_2 group is attached directly to an aromatic ring react directly with retinene$_2$ to exhibit $\lambda_{max.}$ 530–560 mμ. on acidification. It is not necessary to add alkali first. Typical absorption curves are shown in Figs. 1 and 2 and values of $\lambda_{max.}$ are given in Table 1.

Interaction of retinene$_2$ and concentrated acids

(*a*) A solution of retinene$_2$ in $CHCl_3$ was cooled to 0° and mixed with an equal volume of conc. H_2SO_4 cooled to 0°. The $CHCl_3$ solution became green ($\lambda_{max.}$ 735 and 705 mμ.) and the acid became red ($\lambda_{max.}$ 570, 525, 470 mμ.). Retinene$_1$ tested similarly gave for the $CHCl_3$ solution $\lambda_{max.}$ 664 mμ. and for the acid solution 570, 525 and 470 mμ.

(*b*) A solution of retinene$_2$ in light petroleum was added to ice-cold syrupy phosphoric acid. The solution became blue