4. The study of the method in the determination of the isoleucine/leucine ratio in the leucine fraction from the hydrolysates of casein, insulin and edestin gave results which agree with those obtained by microbiological methods except in the case of edestin where there is a very large discrepancy.

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# Studies on Vitamin A

## 5. THE PREPARATION OF RETINENE$_1$—VITAMIN A ALDEHYDE

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The state of knowledge concerning photoreception and the role of rhodopsin (visual purple) has been surveyed recently (Granit, 1947) so that only a brief introduction is necessary here.

Wald (1935–6) found that dark-adapted retinas, which are pinkish purple in colour owing to the presence of relatively large amounts of rhodopsin, turn yellow on illumination. Light petroleum will extract from retinas exposed to light a material which Wald called *retinene*. The petroleum solution, freed from solid matter by centrifuging, shows an ultraviolet-absorption maximum near 365 mμ., displaced to 385 mμ. when chloroform is the solvent. When the antimony trichloride reagent, used in vitamin A studies, is added to a chloroform solution of retinene, a blue colour is produced. The blue solution exhibits a well-defined absorption band with a maximum at 664 mμ. Wald postulated for scotopic vision a cyclic process into which entered vitamin A, retinene and visual purple.

In later studies, Wald (1937, 1938–9 *a*, *b*) found that a similar extraction procedure applied to retinas of fresh-water fish, in which rhodopsin is largely replaced by porphyropsin (visual violet), yielded retinene$_2$. This substance is similar to retinene$_1$ but its absorption spectrum is slightly displaced in the direction of longer wave-lengths, as also is the characteristic absorption maximum of the colour test. The discovery that vitamin A$_2$ (Edisbury, Morton & Simpkins, 1937; Lederer & Rosanova, 1937) tends to take the place of vitamin A in fresh-water fish as compared with sea fish and mammals indicated that retinene$_2$ bears the same relationship to vitamin A$_2$ as retinene$_1$ to vitamin A. This is not surprising, as there was already ample evidence of a connexion between vitamin A deficiency and night blindness associated with retarded formation of rhodopsin (visual purple) in the dark-adaptation process.

The discovery of the retinenes was a notable advance, but paucity of material has delayed its exploitation. The amounts obtainable from eyes are too minute to permit orthodox constitutional investigations and for nearly a decade the substances have remained known only by qualitative spectroscopic characteristics quoted in Table 1.

Table 1. *Absorption maxima of substances concerned in visual processes*

| Substance | $\lambda_{max.}$ (mμ.) | Solvent | $SbCl_3$ colour test (in chloroform) $\lambda_{max.}$ (mμ.) |
|---|---|---|---|
| Vitamin A | 326 | *cyclo*Hexane | 617 inflexion 582 |
| Vitamin A$_2$ | 345–350 | *cyclo*Hexane | 693 |
| Rhodopsin (visual purple) | 502 | 1% aqueous digitonin | |
| Porphyropsin | 520–530 | 1% aqueous digitonin | |
| Retinene$_1$ | 369·5 | Light petroleum | 664 |
| | 389 | Chloroform | |
| Retinene$_2$ | 390 | Petrol | >705 |
| | 405 | Chloroform | |

The conclusion drawn by Wald, that retinene$_1$ is a carotenoid and rhodopsin a conjugated protein with retinene as its prosthetic group, has been much quoted, but it is rather insecurely based. Some writers use the term 'carotenoid' as a class designation for highly coloured $C_{40}$ polyenes including those containing hydroxyl and carboxyl groups. Others include in the class $C_{20}$ polyenes such as vitamin A, which are almost colourless. It is arguable that this loose use of the word carotenoid is permissible, but the suggestion that retinene is itself the prosthetic grouping of visual purple is difficult to reconcile with the displacement of the 369 m$\mu$. maximum of retinene to 502 m$\mu$. in visual purple.

No questions of terminology or tentative interpretation can, however, weaken Wald's demonstration that the vitamins A and the visual pigments are somehow linked through the retinenes. From a chemical standpoint, the preparation of the pure retinenes, the elucidation of their structures, and the changes they can be made to undergo are major objectives in the study of visual processes.

Morton (1944), in a survey of the problem based primarily on the interpretation of absorption spectra, came to the conclusion that retinene$_1$ must be the aldehyde of vitamin A. By far the most plausible explanation of the 369 m$\mu$. absorption maximum of retinene$_1$ was the replacement of $CH_2OH$ in vitamin A by CHO, as this would give six conjugated double bonds instead of five, and so account for the displacement from 326 to 369 m$\mu$. Oxidation at any other point in the vitamin A molecule could not fail to displace the absorption in the opposite direction. Similar arguments were valid for the retinene$_2$-vitamin A$_2$ system. The task of preparing such an aldehyde seemed formidable because of the five conjugated double bonds present in the vitamin A molecule. It was not easy to see how ordinary oxidative processes could be restricted to the terminal $CH_2OH$ group and a study of the literature showed that in the Oppenauer reaction any aldehyde formed would tend to undergo further change. Considerations of that kind did not, however, dispose of the matter.

Direct oxidation of vitamin A might be expected to result in simultaneous attack upon the terminal $CH_2OH$ group and upon the double bonds, yielding a variety of products, but the possibility that a little vitamin A aldehyde might be formed, which would escape further attack, seemed worthy of investigation, especially as chromatographic adsorption gave some promise of a separation of the mixture. Morton & Goodwin (1944), by shaking vitamin A concentrates dissolved in light petroleum with dilute aqueous $KMnO_4$ in the presence of a little $H_2SO_4$ and chromatographic separation of the products, always obtained some fractions which showed an absorption band at 365–370 m$\mu$. in saturated hydrocarbon solvents and at 385 m$\mu$. in chloroform. In the latter solvent the solute gave a 664 m$\mu$. maximum with the antimony trichloride reagent. The yields of spectroscopically homogeneous material were small, but there could be no doubt that retinene was an oxidation product of vitamin A, and that it was very probably the simple aldehyde.

The suggestion that retinene was vitamin A aldehyde was soon confirmed by Hunter & Hawkins (1944), who obtained from vitamin A by the Oppenauer reaction a material showing two absorption maxima at 350 and 368 m$\mu$. respectively, and giving a colour-test maximum at 657 m$\mu$. It also yielded a 2:4-dinitrophenylhydrazone, m.p. 207–209°, and was converted to vitamin A by the Pondorff reduction. Hunter & Hawkins were kind enough to allow two of us to test their product. The difference between 657 and 664 m$\mu$. turned out to be a matter of spectroscopic technique only, but the presence of an ultraviolet maximum at 350 m$\mu$. was not compatible with homogeneity. Their material, though rich in retinene, was clearly impure.

A recent preliminary paper by van Dorp & Arens (1947) describes an alternative synthesis of vitamin A aldehyde in impure form.

Orientating experiments in which the present authors were assisted by S. Roberts showed that potassium permanganate was the most suitable oxidizing agent and that variables such as temperature, concentration of reactants, relative volumes of phases, emulsification, etc. were of importance. Some retinene was always obtained, but yields were generally poor and not very reproducible. The one consistent tendency seemed to be an improved yield when hydrated manganese dioxide was formed, i.e. when the sulphuric acid used was insufficient to give a clear solution. An apparatus was then made in which small droplets of a solution of vitamin A in light petroleum were allowed to ascend a tall column of permanganate solution. Reaction between vitamin A and permanganate was very sluggish and it seemed possible that retinene could not be formed until some manganese dioxide was present.

Some years before Wald discovered retinene, one of us in collaboration with Sir Jack Drummond had studied the fate of vitamin A concentrates left to stand over various solids. Nothing came of the work at the time, but re-examination of the working notebooks showed that a colour-test maximum had been seen at 664 m$\mu$. in a solution which had been in contact with manganese dioxide. A feeble band in the same position had been seen in tests with residues from the purification of carotene. At that time the mechanism of the antimony trichloride colour test was even more uncertain than it is now, and a maximum at 664 m$\mu$. conveyed no more than a hint of some unknown impurity or artefact. When, however, the 664 m$\mu$. band became a 'label' for retinene, the old observations slipped into a pattern. It was therefore decided to investigate the adsorption of vitamin A from inert solvents on solid oxidizing agents, in the hope of limiting the attack to the primary-alcohol grouping.

The outcome has been a smooth conversion to retinene at room temperature when vitamin A in light petroleum was allowed to stand over manganese dioxide.

## EXPERIMENTAL

### *Materials and apparatus*

*Vitamin A.* Two types of preparation were used:

(1) A vitamin A alcohol concentrate, from the British Drug Houses Ltd., of $E^{1\%}_{1\,cm.}$ 325 m$\mu$. 600 (*c.* 1,000,000 i.u./g.).

(2) A vitamin concentrate prepared in the laboratory from a rich halibut-liver oil. The non-saponifiable matter was dissolved in methanol and most of the sterols were crystallized out at −35°. The filtrate was freed from solvent and the residue showed $E^{1\%}_{1\,cm.}$ 326 mμ. 900, indicating the presence of more than 50% vitamin A alcohol.

*Manganese dioxide.* Three types of material were used:

*Sample A* was the ordinary granular laboratory reagent.

*Sample B* was a much finer grade of the commercial product, but differed from *A* only in the size of the granules.

*Sample C* was prepared by mixing aqueous solutions of equivalent amounts of $MnSO_4$ and $KMnO_4$, filtering, washing until free from sulphate ions and drying on a porous plate in a desiccator.

*Light petroleum.* The ordinary material, b.p. 40–60°, was mainly used, but for some of the work a 60–80° fraction purified for spectroscopy was substituted.

*Alumina.* Small quantities of 'Brockman' activated alumina (Merck) were at first available. A material purchased from Savory and Moore Ltd. proved satisfactory, but the greater part of the work was carried out using Grade 0 activated alumina (Peter Spence and Co.) weakened by incorporation of water to the extent of 10% (w/w) just before preparing the column.

*Antimony trichloride reagent.* For most of the work a saturated solution of anhydrous antimony trichloride in B.P. chloroform was used, the solvent having been previously left to stand over $CaCl_2$ to remove ethanol.

Ultraviolet-absorption spectra were determined by means of the Beckman quartz photoelectric spectrophotometer and the colour-test observations were made visually using a Hilger-Nutting spectrophotometer.

### *Preparation of retinene*

A convenient amount (0·2 g.) of vitamin A concentrate ($E^{1\%}_{1\,cm.}$ 325 mμ. 600) is dissolved in light petroleum (100 ml.) in a 250 ml. conical flask, and a calculated quantity of manganese dioxide (5 g. sample *A*) added. The weight of oxide to be used depends upon its state of subdivision, and is arrived at by a few small-scale preliminary experiments. The flask is tightly corked to prevent escape of solvent and is left in the dark at room temperature. The mixture is shaken occasionally (but this is not essential) and at the end of 6–10 days the transformation to retinene is almost complete. Longer periods of standing reduce the yield of retinene. The solution is now filtered and the manganese dioxide is washed once with light petroleum. Yields up to 80% calculated on the vitamin A used have been obtained. For example, in one experiment the product exhibited an almost symmetrical absorption curve with a maximum at 368 mμ. ($E^{1\%}_{1\,cm.}$ 390). With the antimony trichloride reagent a blue solution was obtained, $\lambda_{max.}$ 664 mμ. By applying the correction procedure of Morton & Stubbs (1946) the contribution attributable to unchanged vitamin A was $E^{1\%}_{1\,cm.}$ 325 mμ. 50, and that due to retinene was $E^{1\%}_{1\,cm.}$ 367 mμ. 382. The retinene is purified by chromatographic adsorption on slightly weakened alumina (*v.s.*), and fractions exhibiting $E^{1\%}_{1\,cm.}$ 365 mμ. 1250 (in light petroleum) are regularly obtainable.

As the maximum extinction coefficients of vitamin A and retinene do not differ greatly in light petroleum, the results imply 75% conversion and contamination of the product by about 10% of unchanged vitamin A.

*The effect of agitation.* A 0·5 g. portion of a concentrate, containing about 0·16 g. vitamin A alcohol, was dissolved in 250 ml. light petroleum; 100 ml. of the solution and 5 g. $MnO_2$ (sample *A*) were placed in a glass-stoppered bottle of 250 ml. capacity. The mixture was subjected to brisk but not violent agitation on a mechanical shaker for 8 hr. daily. A similar mixture was left to stand in the dark without shaking. After 6 days, measured portions of the two solutions were removed, and the blue solutions obtained with the antimony trichloride reagent were studied quantitatively using the wavelengths 617 and 664 mμ. characteristic respectively of vitamin A and retinene and afforded the following results:

| | $E^{1\%}_{1\,cm.}$ at 617 mμ. | $E^{1\%}_{1\,cm.}$ at 664 mμ. |
|---|---|---|
| Without agitation | 754 | 554 |
| With agitation | 768 | 710 |

Agitation thus resulted in an appreciably improved yield of retinene. The apparent increase in vitamin A in the second case was due to the fact that, although the retinene colour-test absorption band shows a sharp maximum near 664 mμ., there is also considerable absorption at 617 mμ. This becomes superimposed upon that from residual vitamin A, so that the intensity of absorption at 617 mμ. leads to an overestimate of the vitamin A present.

The effect of agitation is reproducible, but one further experiment requires to be mentioned. In this, a Waring Blendor was used to secure extremely vigorous agitation. Under normal conditions several hours are required for the appearance of the retinene spectrum, but in this case considerable amounts of retinene appeared within 1·5 hr. The heat generated by the very rapid stirring caused much evaporation of the light petroleum. The use of the Blendor was discontinued as being unnecessarily hazardous. For work on a larger scale, vigorous stirring in a suitably designed apparatus may well be worth while.

*Rate of production of retinene.* Many experiments on the time factor were carried out. The following is typical: 0·2 g. of concentrate (0·06 g. vitamin A) was dissolved in 100 ml. of purified light petroleum and the solution was left over manganese dioxide (5 g. sample *A*) in the dark. Sample portions were withdrawn at intervals, appropriately diluted, and examined spectrophotometrically. The curves shown in Figs. 1–4 exhibit a steady decrease in the intensity

of the vitamin A maximum with a corresponding increase in absorption at 369 m$\mu$.

The reaction can also be followed spectrographically. Figs. 1–4 indicate how the absorption spectrum of a test solution gradually widens and moves towards longer wave lengths as retinene becomes the predominating solute. Applying the correction procedure (Morton & Stubbs, 1946) to these curves, the change in vitamin A concentration in the solution can be determined. Using data obtained for crystalline retinene a similar correction can be used to compute the corresponding retinene concentration. The sum of the two computed curves is not identical with the observed curve. The difference curve is wide and of indeterminate structure; its origin is unknown.

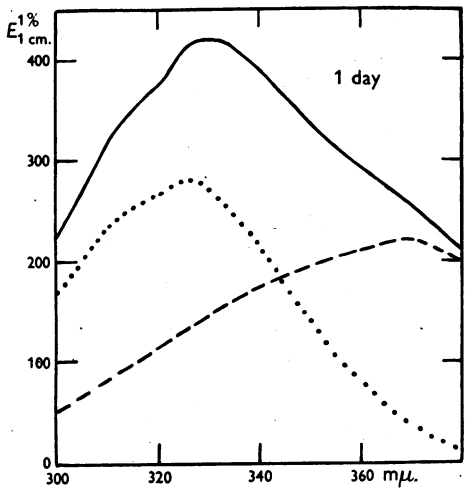



Fig. 1.

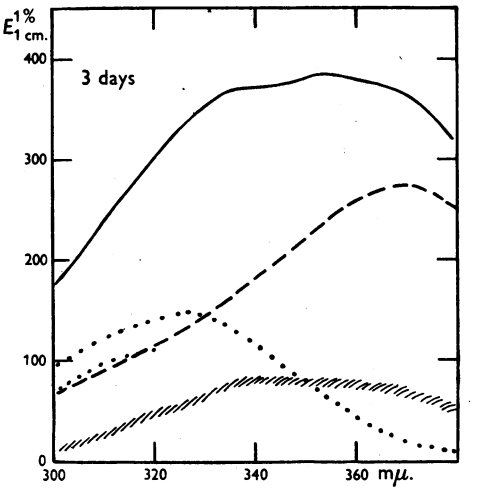



Fig. 2.

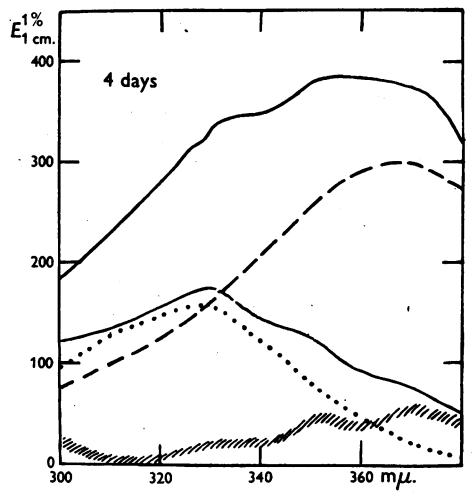



Fig. 3.

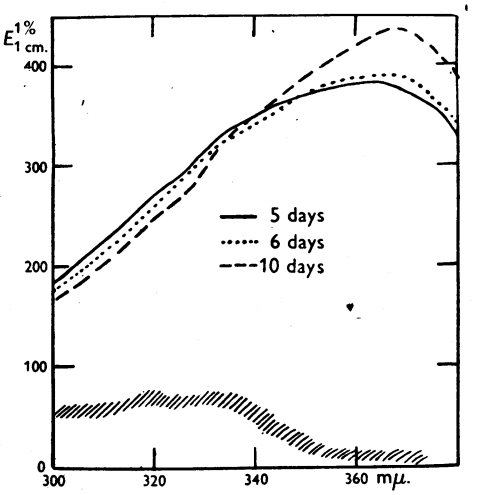



Fig. 4.

Figs. 1–4. Absorption curves obtained when a vitamin A concentrate is left over manganese dioxide in the dark (solvent, light petroleum) for periods of 1–10 days. The vitamin A band is gradually replaced by the absorption due to retinene$_1$ (measurements at intervals of 5 m$\mu$. except around maxima where intervals were 0·5 m$\mu$.). Observed absorption ——; vitamin A by correction procedure · · · · ·; retinene by correction procedure - - - - - -; residual absorption after correcting for vitamin A and retinene //////.

*Relative amounts of vitamin A, manganese dioxide and petroleum.* The amount of manganese dioxide required for the best yield is critical and depends upon two factors: (*a*) the state of subdivision of the

sample, and (*b*) the absolute amount of vitamin A in the reacting solution. Tests have been made with three different samples and good results have been obtained with each. It is, however, clear that the finer the state of subdivision, the smaller is the amount of oxide needed for the maximum yield of retinene. Thus with 1 g. of concentrate (0·3 g. vitamin) in 100 ml. of light petroleum, the best results were obtained with 25 g. of sample *A*, 5 g. of sample *B* and 1 g. of sample *C* (*v.s.*). The use of too little manganese dioxide makes the reaction slower than it need be, and the final yield is then below the best. Too much oxide also adversely affects the yield. Table 2 records an experiment in which solutions of a vitamin A concentrate in light petroleum (purified for spectroscopy) were prepared and 100 ml. portions left over manganese dioxide in the dark.

Table 2. *Effect of vitamin A concentration and amount of manganese dioxide on yield of retinene*

| Vitamin A concentrate in petroleum (%) | Manganese dioxide | | $E^{1\%}_{1\,cm.}$ 664 mμ. after 6 days |
|---|---|---|---|
| | Wt. (g.) | Sample | |
| 0·2 | 5 | *A* | 756 |
| 0·2 | 5 | *B* | 335 |
| 0·5 | 5 | *A* | 493 |
| 1·0 | 5 | *A* | 393 |
| 1·0 | 25 | *A* | 702 |

These and other experiments show that for a given sample of manganese dioxide the ratio weight of manganese dioxide/weight of vitamin A is of great importance. The actual concentration of vitamin A (i.e. the amount of solvent) used was found to be much less critical.

*The effects of light and of temperature.* Light exerts a deleterious effect for both vitamin A and retinene are slowly destroyed in diffuse daylight. Experiments carried out in ordinary glassware kept away from direct sunlight gave very poor yields of retinene compared with similar experiments where exposure to daylight was reduced to a minimum. Other tests in which the reaction mixture was kept at 37° in the dark gave extremely poor results compared with control experiments carried out at room temperature. Warmth and light are therefore to be avoided.

*Substitution of other oxidizing agents. Replacement of light petroleum by diethyl ether.* Various solid oxidizing agents were tried as substitutes for manganese dioxide, but in no case could any retinene be observed. Chromium sesquioxide, chromic oxide, barium peroxide, lead peroxide, lead tetroxide and silver oxide all proved useless, and when portions of each were mixed with manganese dioxide, the yield of retinene could be accounted for by the $MnO_2$ moiety.

If vitamin A in light petroleum is left for long periods (e.g. 35 days) over solid oxidizing agents, other than manganese dioxide, vitamin A tends to disappear and to be replaced by a product, not retinene, exhibiting an absorption maximum near 280 mμ.

When purified diethyl ether was tried as solvent instead of light petroleum, the sole reaction product identified (and that in poor yield) was cyclized or anhydro-vitamin A.

*Specificity of the oxidation.* Manganese dioxide was left in the dark with light petroleum solutions of *iso*propyl alcohol, benzyl alcohol, octadecyl alcohol, cetyl alcohol and a mixture of $C_{20}$ alcohols kindly supplied by Prof. T. P. Hilditch. In no case did chemical or spectroscopic tests for aldehydes give positive results.

Solutions of vitamin A esters in light petroleum showed little change after standing over manganese dioxide.

Ergosterol undergoes changes not yet fully elucidated, but calciferol is not readily attacked.

### *Purification of retinene*

When the interaction between manganese dioxide and vitamin A is almost completed the mixture is filtered and the filtrate is poured on to a column of activated alumina. The best results are obtained in our experience by the use of slightly weakened alumina prepared as previously described. The chromatogram is developed by means of light petroleum:

*Fraction* 1. A yellow zone passes quickly down the column. The material gives a 617 mμ. band with the $SbCl_3$ reagent. It may contain a small amount of cyclized vitamin A and any vitamin A ester present in the original concentrate.

*Fraction* 2 travels more slowly as a broad brown zone containing most of the retinene.

*Fraction* 3 is a more strongly held pale-yellow zone. With the $SbCl_3$ reagent the eluate from this zone shows maxima at 617 and 664 mμ.

*Fraction* 4 is very firmly adsorbed. It remains as a brown zone at the top of the column and can only be removed by elution with ether or chloroform. The fraction shows no characteristic ultraviolet-absorption spectrum and the dirty red solution formed with the $SbCl_3$ reagent exhibits no well-defined maximum.

Fraction 2 is now reduced to small bulk by distilling off much of the solvent. The concentrated solution is rechromatographed on weakened alumina. The retinene fraction is collected and examined spectroscopically. The ultraviolet absorption is maximal at 369 mμ. (in petroleum) and a value of $E^{1\%}_{1\,cm.}$ 1250 is regularly obtained. This seems to be the limit of purification attainable by simple chromatography.

It is important that the alumina should not be too active. Ordinary 'Brockman' alumina is satisfactory but the solvent percolates rather slowly, whilst the grade 0 (Peter Spence and Co.) alumina as supplied tends to produce a red artefact from retinene ($\lambda_{max.}$ 290 m$\mu$.; $SbCl_3$ test $\lambda_{max.}$ 572→610 m$\mu$.). By using weakened alumina the chromatography is speeded up and decomposition of retinene is eliminated.

*Examination of used manganese dioxide.* After filtering the reaction mixture, the manganese dioxide retains some adsorbed material which can be eluted with chloroform. The solution gives a blue colour with the Carr-Price reagent with maxima at 573, 610 and 664 m$\mu$. If the chloroform is removed and replaced by light petroleum, ultraviolet maxima at 290 and 365 m$\mu$. are recorded. It seems that some retinene and some of the red artefact are adsorbed on the manganese dioxide.

### *Crystallization of retinene*

Purified light petroleum was found to be the best solvent for crystallization. The solute was a retinene fraction $E^{1\%}_{1\,cm.}$ 369 m$\mu$. 1250 and a concentration of about 5% (w/v) was used for a preliminary cooling to −72° for 48 hr., when a small yield of feathery white crystals was obtained. This material (which was discarded) showed an absorption band at 270 m$\mu$. in light petroleum or in chloroform, and with the $SbCl_3$ reagent a rose-coloured solution was obtained showing continuous end absorption over the whole visible spectrum range.

In addition to the above material very small amounts of a substance (also discarded) with a band of comparatively low intensity at 348 m$\mu$. in petroleum or in chloroform were obtained. The $SbCl_3$ reagent gave a blue-coloured solution with absorption bands of approximately equal intensity at 664 and 617 m$\mu$.

After removal of these impurities by filtering through a Buchner funnel cooled with solid carbon dioxide, the mother liquor was concentrated *in vacuo* to one-fourth its original bulk. The concentrated solution was again cooled to −72° for 24 hr. when a brown semi-solid mass was obtained. The mother liquor was decanted off and the residue dissolved in freshly distilled light petroleum to give a 5% (w/v) solution. This was kept at −72° for 10 days when a large crop of reddish brown crystals, m.p. 56·5–58°, was obtained which showed an absorption band with a single maximum at 373 m$\mu$. in *cyclo*hexane, $E^{1\%}_{1\,cm.}$ 1354.

The material so obtained was recrystallized very carefully from 5% (w/v) solution in light petroleum, the solution being cooled slowly from 0° to −40° over a period of 7 hr. The mixture was then left at −72° for 24 hr. when large orange-red crystals were obtained in good yield. The overall yield (of crystallization) was about 20%. The crystals are large clusters predominantly needle-like in shape and melt at 61–62°. (Found: C, 84·2; H, 9·76%; mol. wt. (Rast) 236. $C_{20}H_{28}O$ requires C, 84·5; H, 9·85%; mol. wt. 284.)

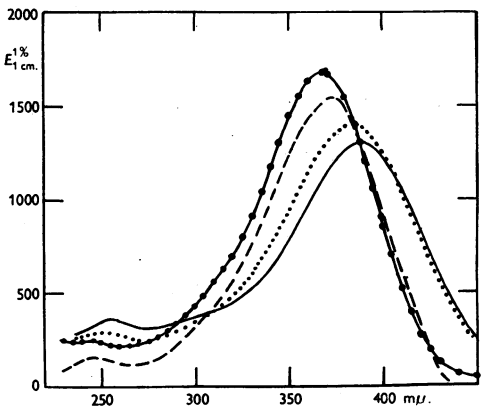



Fig. 5. Absorption spectrum of retinene$_1$ in light petroleum •—•; *cyclo*hexane — — —; ethanol · · · · · ·; and chloroform ——.

Table 3. *Absorption maxima of retinene in various solvents*

| Solvent | $\lambda_{max.}$ (m$\mu$.) | $E^{1\%}_{1\,cm.}$ |
|---|---|---|
| Light petroleum (40–60°) | 369·5 | 1685 |
| *cyclo*Hexane | 373 | 1548 |
| Ethanol | 385·5 | 1400 |
| Chloroform | 389 | 1303 |

The absorption spectra of crystalline retinene have been determined in various solvents. The full curves are recorded in Fig. 5 and the maximum intensity of absorption is recorded in Table 3, e.g. in *cyclo*hexane,

$E^{1\%}_{1\,cm.}$ 373 m$\mu$., 1548; $E^{1\%}_{1\,cm.}$ 245 m$\mu$., 159.

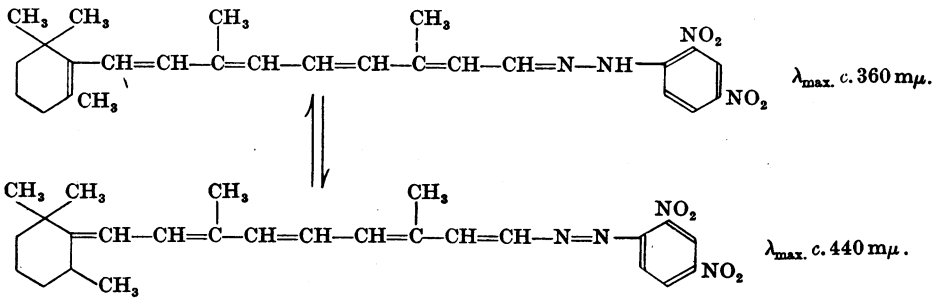
With the antimony trichloride reagent a blue-coloured solution was obtained, giving a single absorption band at 664 m$\mu$., with an $E^{1\%}_{1\,cm.}$ value of 3400.

### *Derivatives of retinene*

*Retinene-2:4-dinitrophenylhydrazone.* An ethanolic solution of 2:4-dinitrophenylhydrazine sulphate was added to an equivalent amount of retinene in ethanol, when an immediate darkening of the solution was observed. From this solution a good crop of crystals was obtained and the material was crystallized from ethanol and from acetone. Reddish brown needles were obtained with m.p. 207–208°. (Found: C, 66·8; H, 6·9; N, 12·2%; mol. wt. (Rast) 336. Calc. for $C_{26}H_{32}N_4O_4$: C, 67·2; H, 6·9; N, 12·1%; mol. wt. 464.) The spectrum of the derivative showed bands at 442 and 260 m$\mu$. with $E^{1\%}_{1\,cm.}$ 1166 and 346 respectively.

*Retinene semicarbazone.* This derivative was prepared by adding an aqueous ethanolic solution of semicarbazide hydrochloride to an ethanolic solution of retinene. The mixture was warmed for a few minutes, and after standing overnight was extracted with ether and the semicarbazone crystallized from a mixture of ether and light petroleum. Although work has not yet been completed on this material, one sample of the derivative, which must have been of high purity, had m.p. 161–164° and an absorption band at 385 m$\mu$. in chloroform with $E^{1\%}_{1\,cm.}$ 1742. On standing in solution at 0° for some weeks, the intensity of the 385 m$\mu$. band had decreased with the production of another band of equal intensity at 365 m$\mu$. These data are at variance with those reported by van Dorp & Arens (1947) who state that this derivative has m.p. 207–209° (with decomposition), an absorption spectrum $\lambda_{max.}$ 375 m$\mu$., log $E_{max.}$ 4·87, and reacts in dilute solutions with chloroformic $SbCl_3$ to give a purple-red colour and in stronger solutions a blue-violet colour. Comparison with our data is difficult for they do not state the solvent used to determine the ultraviolet spectrum and the colour-test statements are too qualitative.

*Retinene hydrazone derivatives.* Two hydrazone derivatives of retinene have been obtained by reaction of a solution of hydrazine hydrate with retinene followed by fractional crystallization. Work has not yet been completed but the following details have been obtained.




*Derivative A*, m.p. 108–109°. Single broad band absorption maximum in ethanolic solution.

$\lambda_{max.}$ 355 m$\mu$.; $E^{1\%}_{1\,cm.}$ 280.

*Derivative B*, m.p. 135–145°. Two absorption bands in ethanolic solution.

$\lambda_{max.}$ 460 m$\mu$.; $E^{1\%}_{1\,cm.}$ 1502
298 m$\mu$.; 322.

With pure retinene, a semicarbazone, m.p. 193–195° and $E^{1\%}_{1\,cm.}$ 385 m$\mu$. 2062, and a 'hydrazone', m.p. 177°, $\lambda\lambda_{max.}$ 462 and 297 m$\mu$., $E^{1\%}_{1\,cm.}$ 1930 and 334 respectively, have since been obtained. The semicarbazone analyzed correctly. Found: C, 73·8; H, 9·1; N, 12·3. Calc. for $C_{21}H_{31}N_3O$: C, 73·0; H, 9·1; N, 12·5%. The '*hydrazone*' analyzed correctly for an azine. Found: C, 85·1; H, 10·0; N, 4·74; mol. wt. 500. $C_{40}H_{56}N_2$ requires C, 85·1; H, 9·9; N, 4·9%; mol. wt. 564.

## DISCUSSION

The preparation described above forms a convenient and economical method of preparing retinene. The material so formed shows the same spectral characteristics as that isolated from retinas by Wald (1935–6), and differs from the materials prepared by Morton & Goodwin (1944) and by Hunter & Hawkins (1944) only in its state of purity. Hunter & Hawkins obtained a material showing absorption bands at 368 and 350 m$\mu$. but it is now apparent that the 350 m$\mu$. band is not characteristic of retinene but is due to an impurity, probably an artefact. This artefact has been isolated in an impure state and in small yield and may possibly be a polymerization product of retinene.

The preparation of the retinene crystals and of its derivatives gives proof to the suggestion of Morton (1944) that retinene is the aldehyde of vitamin A. Some points of doubt, however, still remain to be investigated completely, e.g. the absorption spectrum of retinene-2:4-dinitrophenylhydrazone is atypical, though it is possible that the following equilibrium may exist:

The low molecular weights obtained for this compound and for retinene were obtained by Rast's method, and it is reasonable to believe that these results are due to the rather severe treatment required by this method. A further point of doubt concerns the two hydrazone derivatives, although the spectral evidence indicates that *derivative A* is the normal hydrazone type and *derivative B* is, in all probability, an azine compound of the type

*R*—CH=CH—CH=N—N=CH—CH=CH—*R*.

The semicarbazone derivative prepared seems normal, and the production of the 365 m$\mu$. band on standing in solution can be explained by assuming the existence of the following dynamic equilibrium (see p. 523).

The conversion of vitamin A alcohol to retinene depends upon the surface catalysis of manganese

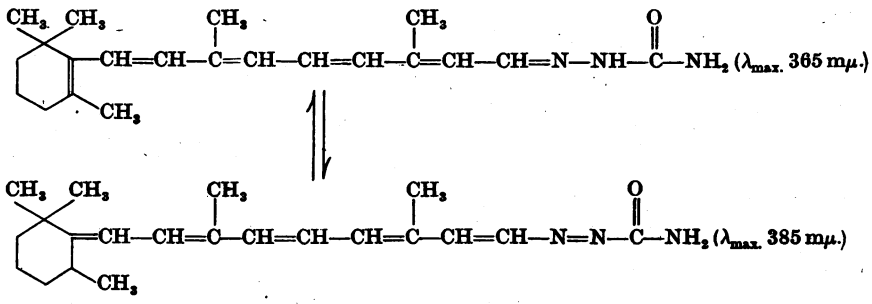
dioxide. In all probability the process is triphasic, the three stages comprising:

(1) The removal of vitamin A alcohol from the light petroleum phase by adsorption on manganese dioxide.

(2) The conversion of the $CH_2OH$ group to the CHO group.

(3) The passage of retinene into the petroleum phase.

The oxidation, as far as our investigations go, is specific, for replacement of either of the reactants




(i.e. vitamin A or manganese dioxide) by a similar substance prevents any comparable reaction. The oxidation process is dependent on two facts, viz. that vitamin A is more strongly adsorbed on manganese dioxide than is retinene, and that the solid adsorbent is capable of converting the primary-alcoholic group to the aldehyde group without attacking other parts of the molecule. Apart from the importance of the reaction from a purely preparative point of view, it may well be that it is of deeper significance. The mechanism connecting vitamin A with retinene in the cycle of scotopic vision is a matter of some doubt, but it is possible that one stage of the retinal process is a surface oxidation analogous to the one described above.

## SUMMARY

1. A simple method of preparing retinene (vitamin A aldehyde) from vitamin A in excellent yield is described.

2. Retinene has been obtained crystalline and fully characterized.

3. The preparation of derivatives of retinene with 2:4-dinitrophenylhydrazine, hydrazine and semicarbazide is described.

4. Absorption spectra of retinene and its derivatives are given.

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# Proteolytic Activity of Preparations of Crystallized Ribonuclease

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The first preparations of ribonuclease were made by methods involving heating (Jones, 1920; Dubos & Thompson, 1938), but since the adoption of the Kunitz (1940) method for the isolation of the enzyme in crystalline form, this step has been omitted. It appears to have been assumed that crystallinity presupposes homogeneity, and crystallized preparations of the enzyme, which have never been exposed to heat, have been used by many workers under conditions in which the presence of other enzymes, and of protease in particular, renders interpretation of results uncertain. It has been established with many proteins that crystallinity is no guarantee of purity (Pirie, 1940) and this seems to apply to ribonuclease. Cohen (1945), when studying the effect of proteolytic enzymes on a