(2) In the presence of ammonia the amino derivatives move much faster than the corresponding hydroxy compounds, but on changing to an acidic solvent this difference is greatly decreased and the relative positions may even be reversed. This is particularly evident in the case of the pyrimidines cytosine and uracil (and their ribosides) in which the reversal takes place at neutrality.

(3) Increase in the number of hydroxyl groups possessed by a substance decreases its rate of movement in all the solvents. This is illustrated by a comparison of the R_F values of hypoxanthine, 6:8-dihydroxypurine and uric acid, and those of adenine and guanine, adenosine and guanosine.

(4) Ribosides move more slowly than the free bases but maintain the same relative positions.

(5) Increasing methylation tends to increase the movement of the substances in all the solvents.

Comparing these results with those of other workers some points of interest may be noted. We find, contrary to Hotchkiss (1948), that, when present in amounts small enough to be completely in solution in the aqueous phase, guanine moves at an appreciable rate in butanol mixtures, and probably corresponds with the 'epiguanine' to which he refers. This is probably due to the fact that his method has too poor a resolution to give a satisfactory picture of the chromatogram as a whole.

Except in acidic solvents, uracil always runs in our chromatograms more slowly than adenine, contrary to the observations of Vischer & Chargaff (1948*a*). These and other minor discrepancies would appear to be due, in part at least, to the different filter paper used by these authors.

SUMMARY

1. A micromethod for the detection and estimation of purines, pyrimidines and related substances is described. It is based upon the detection of spots of these compounds on paper chromatograms by means of a simple contact-printing technique using photographic paper and filtered ultraviolet light, and the sensitivity is such that a few micrograms may be detected.

2. The R_F values of a number of compounds in several solvents are tabulated.

3. Certain generalizations are made correlating chemical structure with the chromatographic behaviour of these substances.

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Studies in Vitamin A

10. VITAMIN A_1 AND RETINENE$_1$ IN RELATION TO PHOTOPIC VISION

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Scotopic (or low intensity) vision is related to the activity of rods, and photopic (or daylight) vision operates by means of cone receptors. When light falls only on rods there is no sensation of colour, and the spectral distribution of sensitivity in scotopic vision can be interpreted in terms of one receptor substance, rhodopsin. For photopic vision, however, the phenomena of colour sensation cannot be explained on the basis of a single absorbing entity with a spectrum corresponding to the photopic sensitivity curve. Nevertheless, the full cone response curve obtained by electrophysiological methods is very similar to the normal photopic sensitivity curve.

One interpretation of the Young-Helmholtz theory is that it implies the existence of three selectively absorbing substances sensitive to different spectral ranges. Many workers have attempted to measure the responses of different receptors to different wavelengths. The usual procedure was first to expose the eye to filtered (or better monochromatic) light in the hope that the receptor affected would be put out of action by photochemical exhaustion. The response of another receptor to

wavelengths of monochromatic light within its own range of sensitivity could then be determined. Prolonged exposure of the eye to one wavelength, however, always produced a reduced response to all wavelengths.

Since 1936, Granit has studied the problem by electrophysiological methods (for review see Granit, 1947). The complicating rod phenomena were eliminated by pretreatment of the eye with light sufficiently intense to bleach all the rhodopsin. After removing the lens and cornea, and applying micro-electrodes directly to the retina, Granit was able to pick up responses from individual nerve fibres when monochromatic light fell on the retina, and in that way made fundamental advances in the combined problems of colour sensation and photopic vision. His scotopic action spectra for frog, toad, cat, rabbit, pigeon, guinea pig, fresh-water eel, etc. were very uniform and enabled him to compute the absorption spectrum of rhodopsin. With light-adapted eyes striking results were obtained. Sensitivity curves widely different from the normal photopic sensitivity curve were often recorded, and as a result of many different series of experiments Granit advanced his dominator-modulator theory.

A dominator is a sensory mechanism, whether scotopic or photopic, characterized by a broad sensitivity curve and making available for vision a large range of wavelengths. The differences between the photopic and scotopic dominators account for the Purkinje shift, and the scotopic dominators correspond with rhodopsin or porphyropsin, depending upon whether the eye under study makes use of vitamin A_1 or vitamin A_2. The fact that the photopic dominator response curves are always displaced by some 60–70 mμ. in the direction of longer wavelengths compared with the corresponding scotopic curves whether the eyes contain vitamin A_1 or A_2 or both, suggests that the receptor substances may all be related to the vitamins A. The dominator curves are concerned with the sensation of brightness and are too broad to allow them an important role in colour discrimination.

A modulator, on the other hand, is characterized by a much narrower response curve. The substantial body of data accumulated by Granit shows that the modulator action spectra fall into groups situated in three spectral regions: 440–470, 520–540 and 580–600 mμ. There is an added complication in the existence of a light-sensitive modulator near 500 mμ. The most light-resistant modulator appears to be that near 600 mμ. The narrow action spectra make the modulators peculiarly suitable for colour sensitivity. There is independent evidence that colour mechanisms are not responsible for brightness discrimination (Wright & Granit, 1938).

The outstanding implication of Granit's work, from the present point of view, is that his action spectra provide serviceable 'labels' for hypothetical light-absorbing substances concerned in vision, provided that the relationship between absorption spectra and action spectra is borne in mind (see Ball, Collins, Morton & Stubbs, 1948; Dartnall & Goodeve, 1937; Hecht, 1937; Wald, 1938).

Von Studnitz (1932) bleached isolated retinas and measured the changes in light absorption at different wavelengths, but the evidence he produced for a photosensitive cone pigment is technically questionable. He later (von Studnitz, 1937) extracted from frog retinae an ether-soluble material with $\lambda_{max.}$ 560 mμ.; but when his data are replotted on a scale suitable for comparison with photopic sensitivity it seems that the absorption curve was broader than the 'dominator' curve. For the tortoise (*Testudo graeca*) he recorded $\lambda_{max.}$ for the extract at 560 mμ., although the photopic sensitivity maximum is at 600–610 mμ. Using the snake *Tropidonotus* he obtained extracts with absorption maxima at 468, 560 and 650 mμ. Chase (1938) extracted a water-soluble cone substance (absorption maximum 530 mμ.), and Wald (1938) deduced from differential photo-decomposition of chicken retinae an absorption maximum at 575 mμ., which he attributed to a cone pigment, iodopsin. Hosoya, Okita & Akune (1938), using the rod-free retinae of tortoises, claimed to have extracted by means of 2% sodium cholate three water-soluble pigments the absorption spectra of which showed maxima at 460, 570 and 670 mμ. respectively, but the data were not very satisfactory. Hanström (1940) extracted the macular region of monkey retinae and found maxima at 460 and 590 mμ. due to ether-soluble substances.

Bliss (1946*a*, *b*) has confirmed the existence of Wald's iodopsin in chicken retinae and has been able to extract it, although not in a very 'pure' state. The iodopsin was more labile than rhodopsin, and bleaching by light produced retinene in 'large' amount, but there was no detectable change in pH or inorganic phosphate concentration.

That a deficiency of vitamin A impairs scotopic vision is by now familiar, but the relationship of vitamin A to photopic vision is not so clear cut. Inasmuch as there is no real hint of any other key substance in vision, the next step is to find out whether vitamin A is capable of being the only key substance.

Vitamin A and retinene give rise to deep blue solutions with antimony trichloride in chloroform, and the sharpness of the absorption bands, coupled with the transient nature of the blue materials, suggests that under suitable environmental conditions vitamin A or retinene might give rise to 'ionized' or 'halochromic' molecules (see Meunier & Vinet, 1947) resembling the modulators of Granit. When vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid at temperatures near 0°, coloured solutions are produced which exhibit well-defined selective absorption with maxima corresponding closely with Granit's maxima. These results are recorded and discussed below.

EXPERIMENTAL

The materials used were crystalline samples of vitamin A alcohol and retinene$_1$, the ionizing media being H_2SO_4 (sp.gr. 1·84) and H_3PO_4 (sp.gr. 1·75). The Beckman spectrophotometer was used for the quantitative measurement of absorption spectra, the Hilger-Nutting visual instrument for some qualitative determinations.

RESULTS

Vitamin A and sulphuric acid

A few minute crystals of vitamin A were added to about 20 ml. of concentrated sulphuric acid and the mixture was well shaken. The vitamin dissolved easily, and a transient purple colour was immediately formed ($\lambda_{max.}$ 620 mμ.). The blue component of the colour very quickly faded, and the solution became bright red with absorption maxima at 465, 520 and 580 mμ. By means of the Hilger-Nutting instrument the diminution in intensity and disappearance of the 620 mμ. band with concomitant appearance and increasing intensity of the 580 mμ. band could be observed, showing clearly an interrelationship between the substances responsible for these absorption maxima. The 520 mμ. band was also unstable and disappeared fairly rapidly at room temperature even in the dark. If the sulphuric acid solution was kept in the solid state at $-78°$ in the dark, all three bands persisted for several days. Under such conditions, i.e. dissolution of vitamin A in very cold sulphuric acid, an absorption band was found at 560 mμ. instead of 580 mμ.

When, immediately after mixing, the sulphuric acid solution of vitamin A was agitated with light petroleum, *cyclo*hexane or ether, coloured materials did not pass into the organic solvent and ultraviolet absorption measurements on the extracts showed only the presence of unchanged vitamin A. When chloroform was used as the extracting solvent a cloudy solution was obtained which developed a pink colour on standing. The absorption spectrum of this chloroform extract (Fig. 1) showed bands at 460 and 555 mμ., due to separate chemical entities, since the absorption bands disappeared at different rates under the influence of heat or light.

A dilute dispersion of concentrated sulphuric acid in chloroform was prepared by shaking a mixture of chloroform and sulphuric acid vigorously for 10 min. and allowing to settle. The chloroform solution was slightly cloudy and an intense bluish red colour was immediately obtained on addition of vitamin A. The absorption spectrum of this coloured solution (Fig. 2) was complicated and exhibited maxima at 470 and 570 mμ. On standing for 15–25 min. in light or the dark the spectrum was resolved into a series of absorption bands.

A solution of vitamin A in ethanol gave an intense blue colour with sulphuric acid ($\lambda_{max.}$ 620 mμ.), which changed rapidly to a bright red ($\lambda_{max.}$ 560 mμ.). This red colour also disappeared very quickly leaving a dirty brown solution which showed no selective absorption in the visible or ultraviolet.

Attempts were made to fractionate the coloured products of the sulphuric acid reaction, but without much success. Dilution with water at 0°, followed by neutralization with sodium carbonate or barium carbonate produced colourless solutions exhibiting no selective absorption in the ultraviolet. The best results were obtained by mixing solutions of vitamin A in ethanol with sulphuric acid at temperatures just high enough to keep the acid liquid.

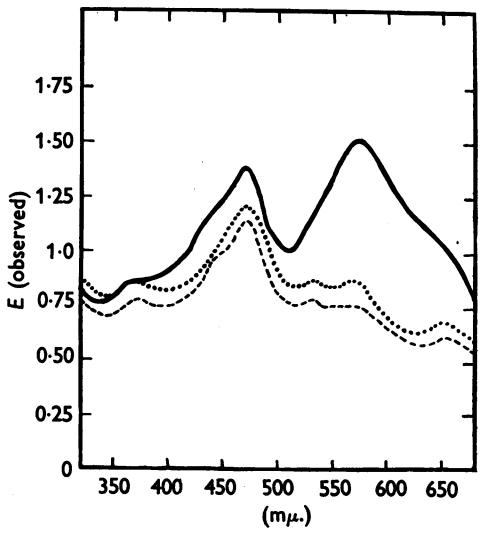


Fig. 1. Absorption spectrum of chloroform extract of a solution of vitamin A in sulphuric acid. ——, measured immediately; - - - - -, after 24 hr. in the dark; . . . . ., after 24 hr. in diffused daylight.

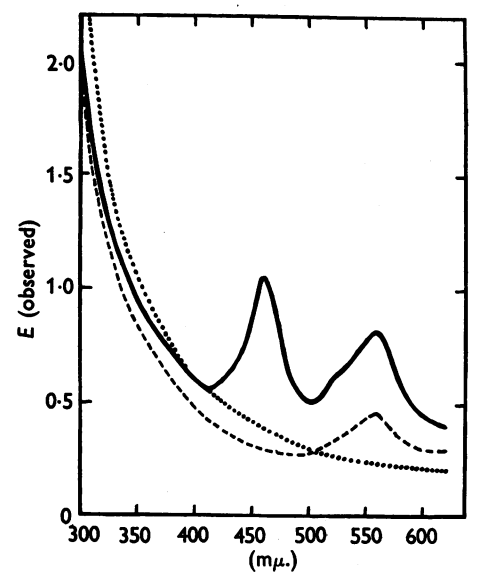


Fig. 2. Absorption spectrum of vitamin A in a chloroform dispersion of sulphuric acid. ——, measured immediately; - - - - -, after 15 min. of diffused daylight; . . . . ., after 25 min. in the dark.

These mixtures were diluted with ice and water, treated with anhydrous sodium carbonate until effervescence ceased, and then extracted with chloroform. The chloroform solutions obtained in this manner were bluish green in colour, the absorption spectra showing maxima at 380 and 465 mμ. The coloured products were stable at −80° in the dark, but faded rapidly on exposure to light or warming to room temperature.

Vitamin A and phosphoric acid

A sample of vitamin A ($E^{1\%}_{1\,cm.}$ 980 at 326 mμ. in *cyclo*hexane) dissolved in a little ethanol, was stirred into phosphoric acid. A blue solution ($\lambda_{max.}$ 620 mμ.) was obtained at first, but rapidly became red with absorption maxima at 480, 540 and 600 mμ. (Fig. 3).

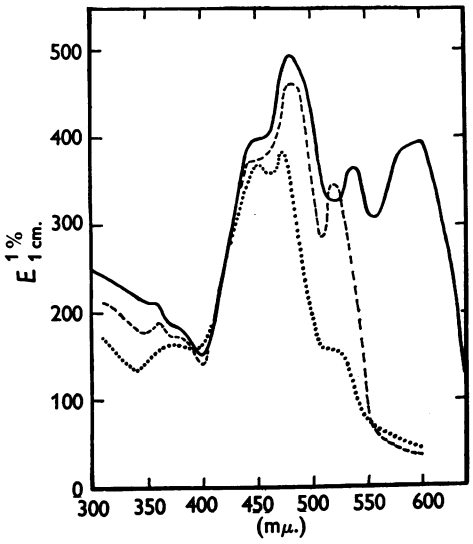


Fig. 3. Absorption spectrum of vitamin A in phosphoric acid. ——, measured immediately; - - - - -, after 5 hr. in the dark; . . . . ., after 5 hr. in diffused daylight.

The 600 mμ. maximum disappeared quickly at room temperature in the dark, but the 480 mμ. band persisted under these conditions. However, the 480 mμ. band decreased in intensity on exposing the solution to light, and after 5 hr. absorption bands of approximately equal intensity were found at 450 and 470 mμ. After 5 hr. in the dark, the 540 mμ. maximum was displaced to 520 mμ., while a similar sample exposed to the light showed only an inflexion at 520 mμ.

Retinene and sulphuric acid

If retinene is dissolved in chloroform and the solution shaken with sulphuric acid, the acid layer becomes bright red in colour and the chloroform deep blue ($\lambda_{max.}$ 664 mμ.). Using light petroleum as the solvent for retinene, no colour develops in the petroleum phase, although the acid layer again becomes red. Treatment of solid retinene with sulphuric acid gave a purplish red solution, the blue component of which disappeared rapidly, leaving a red colour. A complex absorption spectrum was observed with narrow absorption bands at 380, 440, 460, 520 and 560 mμ. (Fig. 4). These absorption maxima disappeared rapidly at room temperature, but persisted well at −80°. After dilution with ice and water, the acid was neutralized with anhydrous sodium carbonate and the solution shaken with chloroform. A colourless chloroform extract was obtained, the spectrum showing no selective absorption in the ultraviolet region. When this chloroform solution was treated with the antimony trichloride reagent a red coloration was obtained,

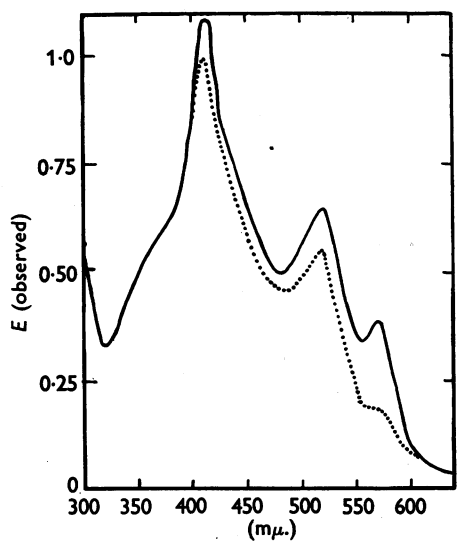


Fig. 4. Absorption spectrum of retinene in sulphuric acid. ——, measured immediately; . . . . ., after 5 min. in diffused daylight.

the absorption spectrum showing maxima at 495, 525 and 590 mμ.

When a solution of retinene in ethanol was stirred into sulphuric acid, the temperature being just above the melting point of the acid, an intense blue colour was obtained ($\lambda_{max.}$ 664 mμ.). This rapidly changed to ruby-red, absorption maxima being observed at 420, 520 and 570 mμ. The solution was diluted, neutralized and extracted as described above; the chloroform extract showed no selective absorption in the visible and ultraviolet regions of the spectrum. With the antimony trichloride reagent a red solution was obtained, the absorption spectrum of which showed bands at 495, 525 and 590 mμ. as above. In the original acid solution it was observed that exposure to light reduced the intensity of each

absorption band, the 570 mμ. maximum diminishing more rapidly than the others. After 5 min. in diffuse daylight the 570 mμ. band had disappeared, whereas the other bands were still quite intense.

With sulphuric acid dispersed in chloroform retinene gave a blue solution ($\lambda_{max.}$ 664 mμ.) which soon became red. The absorption spectrum then exhibited maxima at 390, 515 and 560 mμ., all three bands being reduced in intensity by exposure to light or standing in the dark at room temperature.

Retinene and phosphoric acid

Solid retinene dissolved readily in syrupy phosphoric acid to produce a red solution, a transient blue colour being formed first. The coloured substances were markedly unstable unless the solution was kept at −80°, but increased stability was achieved by dissolving the retinene in a little ethanol

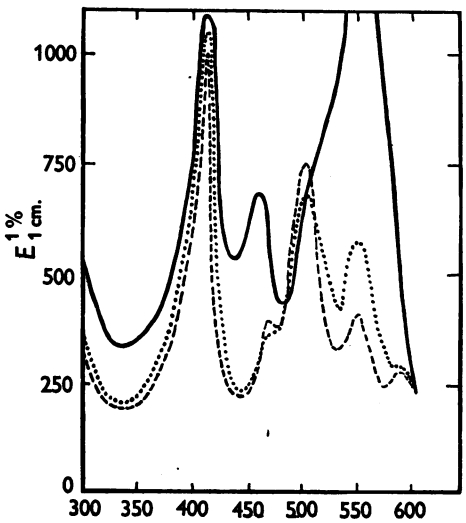


Fig. 5. Absorption spectrum of retinene in phosphoric acid. ——, measured immediately; ....., after 2 hr. in the dark at 0°. - - - - -, after 2 hr. in diffused daylight at room temperature.

before adding the acid. The blue coloration was then recognized as due to a substance with $\lambda_{max.}$ at 664 mμ., and further bands were found at 410, 460 and 560 mμ. in the absorption spectrum measured shortly after mixing (Fig. 5). A pronounced inflexion was observed at 520 mμ. After the solution had been left in the dark at 0° for 2 hr. the absorption spectrum was remeasured. The 560 mμ. maximum had decreased in intensity by more than half, the 460 mμ. maximum had disappeared and the 520 mμ. inflexion had become a sharp maximum at 500 mμ. The 410 mμ. maximum had remained unchanged during this time, and new absorption bands of low intensity were apparently responsible for the pronounced inflexions at 470 and 585 mμ. These changes are recorded in Fig. 5, together with the results obtained when a similar solution was kept at room temperature in diffuse daylight for 2 hr.

A quantitative determination of the intensity of the 560 mμ. maximum was rapidly carried out, the starting material being retinene of $E^{1\%}_{1\,cm.}$ 1400 at 383 mμ. in ethanol. Although the band was decreasing rapidly during the measurement, an $E^{1\%}_{1\,cm.}$ value of 2480 was obtained at 560 mμ. It dropped to 1884 in 3 min., and in 2 hr. there was no maximum at that wavelength. Many similar measurements were made, but it was difficult to distinguish sharply between thermal and photochemical processes. It seems, however, probable that the substances responsible for the 410 and 500 mμ. bands are stable to light even at room temperature, while the 560 and 460 mμ. materials are labile to both heat and light. Examination of the different series of results obtained indicated that the absorption bands at 410–460 mμ., 560 and 664 mμ. are due to separate chemical entities, because they did not appear and disappear together. It is probable, however, that the material responsible for the 664 mμ. band is the precursor of the other substances, since it was always produced first and as the 664 mμ. maximum diminished so the other absorption bands became more prominent.

When solutions of retinene in ethanol are mixed with phosphoric acid, a series of colour changes occurs. The solutions are blue immediately after mixing, and pass through violet and red stages before becoming brown. Attempts made to extract fractions of the coloured materials at different stages by shaking with common organic solvents met with little success. The coloured substances invariably remained in the acid phase. Dilution with ice and water before extraction produced a yellow chloroform extract, the absorption spectrum being flat and featureless, except for a maximum of low intensity at 300 mμ. When this chloroform solution was treated with the $SbCl_3$ reagent a red solution was obtained, the absorption spectrum of which showed intense bands at 475 and 520 mμ., and weak bands at 505 and 535 mμ.

Additional observations. The use of concentrated hydrochloric acid gave results similar to those obtained with phosphoric and sulphuric acids, though in this case the absorption spectra were not measured in detail. However, the same colour changes were noted, i.e. transient blue, passing through intense red to dirty brown, the sequence of changes being very much more rapid with hydrochloric acid than with either of the other acids.

Attempts to fractionate the different materials by chromatography failed, although both kieselguhr and alumina columns adsorbed the coloured products from the chloroform-acid-vitamin A reaction mixture. The substances remained adsorbed in a

narrow zone at the top, irrespective of which organic solvent was used for development. It was found that the colour reactions of vitamin A and retinene with sulphuric acid were inhibited by dilution of the acid with water. In 50 and 80 % (v/v) concentrations, sulphuric acid gives no coloration with vitamin A or retinene.

Table 1. *Comparison of the action spectra of Granit's modulators and the absorption spectra of vitamin A and retinene dissolved in strongly acidic solvents*

(Wavelengths of maximum absorption, $\lambda_{max.}$, in mμ.)

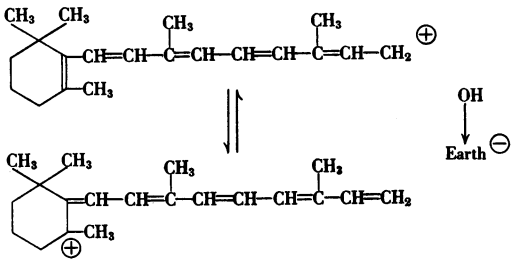
Rhodopsin	—	500	—	—	—	—
Iodopsin	—	—	—	560	—	—
Dominators	—	500	—	560	—	—
Modulators	450–465	500	520–530	—	580–610	—
Vitamin A in conc. H_2SO_4	465	—	520–530	—	590	620
Vitamin A in conc. H_3PO_4	440–480	—	520	—	—	620
Retinene in conc. H_2SO_4	440–460	—	520	560	—	664
Retinene in conc. H_3PO_4	470	500	—	550	590	664

DISCUSSION

The absorption spectra observed when vitamin A or retinene is dissolved in concentrated sulphuric acid or syrupy phosphoric acid bear a striking resemblance to the action spectra of Granit's modulators (see Granit, 1947). This similarity is obvious from the summary of both sets of results given in Table 1.

Granit's modulator maxima fall roughly into three spectral ranges, and the absorption maxima obtained by the mineral acid reactions also fall into similar groups. Some additions to Granit's maxima must be noted, e.g. the 560 and 550 mμ. bands obtained from retinene.

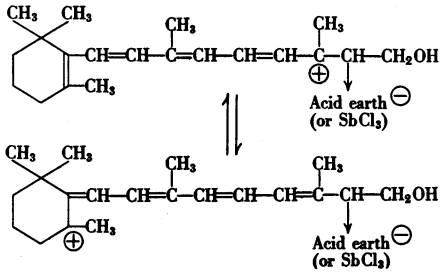
There can be little doubt that the absorption bands obtained are characteristic of 'ionized' or 'halochromic' molecules, as has been postulated by Meunier (1942) for the blue colour obtained when vitamin A is treated with the antimony trichloride reagent. Vitamin A gives a similar blue colour when



adsorbed on certain acid earths, e.g. montmorillonite, and Meunier (1942) suggested that the mechanism was similar to that of the antimony trichloride reaction. These acid earths (or antimony trichloride) possess incomplete electronic octets and are able to accept unshared electrons from the vitamin A molecule, which undergoes polarization and forms positively charged strongly resonating structures.

As a first possibility, Meunier (1942) suggests that the hydroxyl group of vitamin A attaches itself to the acid earth leaving a positively charged carbonium ion. With this type of structure the positive charge would resonate through a system of five conjugated double bonds giving rise to the 620 mμ. maximum.

As a second mechanism Meunier postulated a polarization of the double bond nearest to the primary alcoholic grouping and resonance between the following limiting forms:



With this type of structure the positive charge would resonate through a system of four conjugated double bonds giving rise to the 590 mμ. maximum. Meunier & Vinet (1947), carrying the procedure still further, suggest that resonance of a positive charge through two and three conjugated double bonds would give rise to maxima at 500 and 540 mμ. respectively.

The absorption bands obtained with inorganic reagents are apparently narrower than those implied by Granit's action spectra; this difficulty will be discussed in a later paper.

The photopic receptor substances, presumably conjugated proteins, must be present at the cone surfaces; they must be relatively thermostable as otherwise there would be a spontaneous sensation of light (de Vries, 1948), and when detached from the protein moiety, the halochromic or ionized pros-

thetic grouping derived from vitamin A or retinene would yield no materials not known to be present in the eye.

CONCLUSIONS

The modulator analogues which have been obtained fulfil nearly all the requirements of the colour receptors whose presence is implied by Granit's work, e.g. during fading processes, the maxima fall at different rates, suggesting the presence, not of one product with several bands, but of several products with one maximum to each. The conditions under which these modulator analogues have been produced are obviously unphysiological.

If the problem posed by Granit's modulators is put in the form of a query whether or not the vitamin A-retinene oxidation-reduction system could possibly account for the phenomena observed, the answer is clearly in the affirmative. Which catalysts, old or new, must be invoked before the modulator analogues can be obtained under less obviously unphysiological conditions is a matter for further research.

SUMMARY

1. The absorption spectra of vitamin A and of retinene$_1$ in concentrated sulphuric, phosphoric and hydrochloric acids have been studied. Sharp absorption bands characteristic of unstable ionized molecules were obtained.

2. The results show that vitamin A and retinene$_1$ can give rise *in vitro* to materials simulating the photopic modulators of Granit.

3. The hypothesis that the system vitamin A-retinene$_1$ is important in photopic as well as scotopic vision is strengthened.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance, and to Dr A. L. Stubbs and Dr F. D. Collins for help in the course of the work.

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Studies in Vitamin A

11. REACTIONS OF RETINENE$_1$ WITH AMINO COMPOUNDS

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Rhodopsin or visual purple is a conjugated protein obtained from dark-adapted retinae of many species. The prosthetic group is responsible for the colour ($\lambda_{max.}$ 500 mμ.) and photosensitivity, and is derived from vitamin A or its aldehyde retinene$_1$. No explanation has so far been advanced to account satisfactorily for the displacement of $\lambda_{max.}$ from either 328 mμ. (vitamin A) or 370–390 mμ. (retinene$_1$) to 500 mμ.

The decomposition product of rhodopsin known as indicator yellow shows $\lambda_{max.}$ 440 mμ. in acid solution and 365 mμ. in alkaline solution. Neither the pH sensitivity nor the 440 mμ. maximum has been properly accounted for.

The interaction of purified retinene$_1$ with amino compounds throws some light on these problems. A preliminary account of the work (Ball, Collins, Morton & Stubbs, 1948) has appeared, and the present paper carries the study a stage further.