CAROTENOIDS AND THE VISUAL CYCLE

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The occurrence of vitamin A in the eye tissues (Wald, 1933; 1934–35) is particularly significant because this vitamin is functionally associated with vision. Animals deprived of vitamin A become abnormally insensitive to dim light (night blind), due to failure to synthesize visual purple (Fridericia and Holm, 1925; Tansley, 1931). The present paper examines the nature of this relation and of the visual purple system.

The carotenoids of the eye tissues of several species of frog have been investigated. The pigment epithelium[1] contains large stores of vitamin A and xanthophyll esters. Light liberates from the retinal visual purple a carotenoid, retinene, which is converted by a subse-

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[1] Three distinct tissues line the fundus of the eye. Proceeding posteriorly, these are the retina proper; the dark brown, single layered pigment epithelium, lying in intimate contact with the rods and cones; and the black, vascular choroid. The first two are considered, for histological and physiological reasons, to constitute the retina. In the present paper, the term *retina* designates the retina proper; *epithelium* denotes the pigment epithelium; and this and the choroid are referred to as the *pigmented layers*.

quent thermal reaction to vitamin A. Vitamin A and retinene are also the precursors of visual purple, which they form by combining with a colloidal component, probably protein. The visual processes therefore constitute a cycle.

Franz Boll (1877) first suggested the carotenoid nature of visual purple in the paper that announced its discovery. Boll had observed that in frogs the pigment epithelium forms firm adhesions to the retina when the eye is exposed to light, implying some functional relation between this tissue and the visual process. The pigment epithelium contains golden-colored oil droplets, which fade when the eye is brightly illuminated for long periods. Visual purple, treated with dilute acetic acid, turns a yellow color which Boll believed to be identical with that of the oil droplets. He therefore proposed that the golden pigment may be the stored precursor from which visual purple decomposed by light is continuously re-synthesized.

Boll's associate Capranica (1877) identified the golden pigment with Hoppe-Seyler's "lutein" from the corpus luteum of the cow, and with the yellow pigments of egg yolk, milk, and animal fats, the group of substances now known as carotenoids. Capranica concluded "lutein" to be the parent substance of visual purple.

Kühne and his associates discarded this hypothesis. Ewald and Kühne (1878)[2] showed that visual purple and its acetic acid product possess very different properties from the epithelium pigment. Kühne (1878)[3] also found the latter substance to differ spectroscopically from the pigments of egg yolk (a mixture of xanthophyll and zeaxanthin (Kuhn and Smakula, 1931)) and of the corpus luteum of the cow (β-carotene (Kuhn and Lederer, 1931)). Many animals which possess visual purple lack entirely the epithelium pigment. In frogs its presence in the eye is of no special interest since it is distributed generally throughout the animals' fat deposits (Kühne, 1878).[4] The golden pigment bleaches slowly in bright light, and it is to this property that Kühne ascribed its fading in strongly light adapted eyes (Ewald and Kühne, 1878).[2] This opinion is somewhat obscured by Kühne's additional observation (1879)[5] that the palest epithelium droplets are found, not in the most intensely lighted areas, but just surrounding them.

These observations discredited generally Boll's hypothesis. It had been proposed without conviction since Boll remained undecided whether visual purple was a chemical substance or a physical appearance due to interference phenomena within the rods. It remains the only attempt up to the present to provide a definitive theory of the chemical nature of visual purple and the visual processes.

The identification of vitamin A in the retina revived the possibility that the visual purple system is of carotenoid nature. Haurowitz (1933) has recently

[2] Ewald and Kühne, 1878, Paper II, p. 286.

[3] Kühne, 1878, p. 365.

[4] Kühne, 1878, p. 361.

[5] Kühne, 1879, p. 310.

applied carotenoid tests directly to desiccated visual purple, with negative results. Von Euler and Adler (1934) have attempted unsuccessfully to extract carotenoids from dialyzed and desiccated visual purple. These authors concluded that their experiments offer no support for the carotenoid nature of the visual pigment.

EXPERIMENTS

The frogs used in the present experiments had either been left in total darkness for at least 16 hours (dark adapted) or had been exposed to bright diffuse daylight for at least one-half hour (light adapted). The former were dissected by dim red light, which does not significantly affect visual purple. Light adapted animals were dissected in daylight.

After the frogs had been beheaded and enucleated, the bulbi were opened by cutting around the rim of the sclera. The cornea and lens were lifted off, and the rear half of the eye-ball was dropped into Ringer's solution. The retinas of dark adapted animals were then lifted away from the underlying tissue with a spatula, and were transferred to another container. Adhering bits of pigmented tissue were removed. The pigmented layers also were scooped out of the sclera into Ringer's solution. In light adapted frogs the pigment epithelium adheres to the retina. The basal portions of the pigmented cells may, however, be picked away with fine forceps. These contain all of the pigment epithelium with which the experiments are concerned.

After all the retinas had been prepared and cleared, the various portions of Ringer's solution were combined and centrifuged to yield the total pigmented tissue. Extractions therefore involved in every case the retina proper and the combined pigment epithelium and choroid layer.

Carotenoids of the Pigmented Layers.—The pigmented tissue was washed once with distilled water to remove blood, and was extracted with chloroform or benzine. The extract is of a clear, golden color. It contains two carotenoids:

1. A golden pigment, the spectrum of which is shown in Fig. 1.[6] When dissolved in carbon disulfide it displays absorption maxima at about 445, 476, and 504 mμ. In chloroform the spectrum is of similar form, but displaced so that the maxima occur at 428, 456, and 485 mμ. When shaken with benzine and 90 per cent methanol, the pigment

[6] The spectra shown in Figs. 1 and 3 were measured with the recording photoelectric spectrophotometer of Professor A. C. Hardy at the Color Measurements Laboratory of the Massachusetts Institute of Technology. This instrument has been described by Nutting (*J. Opt. Soc. America*, 1934, **24**, 135). The absorption is plotted as optical density or extinction, log I_o/I, in which I_o is the incident and I the transmitted intensity.

enters the benzine almost quantitatively. After saponification for 3 hours at room temperature in 6 per cent alcoholic KOH, this partition is reversed. Pending further analysis and purification, this substance may be assumed to be an ester of xanthophyll (lutein), $C_{40}H_{54}(OH)_2$ (Kuhn and Winterstein, 1931; Kuhn and Smakula, 1931; Kuhn and Brockmann, 1932).

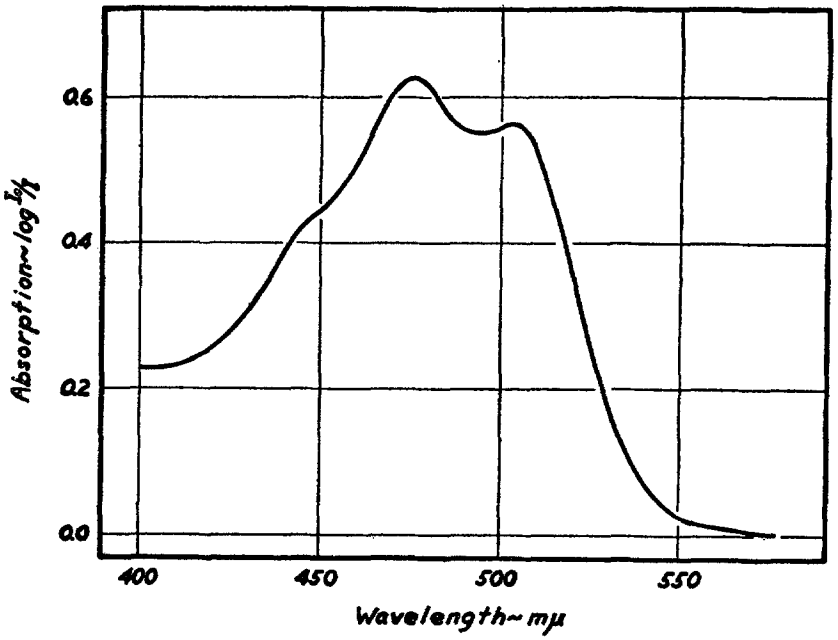

FIG. 1. Absorption spectrum of an extract of pigmented layers in carbon disulfide (*R. catesbiana*). This material had been saponified and partitioned between benzine and 90 per cent methanol; the methanol fraction is shown. Compare with the spectrum of crystalline xanthophyll (Kuhn and Smakula, 1931).

2. A substance which in chloroform solution possesses a single broad absorption band in the ultraviolet at 328 mμ (Fig. 2).[7] With antimony trichloride reagent it yields a deep blue color, due to a single absorption band at about 615 mμ (crude extracts). Partitioned between benzine and 90 per cent methanol, it seeks the benzine layer,

[7] I am indebted to Dr. Elmer Miller of the Chemistry Laboratory, University of Chicago, for measuring this spectrum.

and, like the xanthophyll, reverses this behavior after hydrolysis. These properties identify it as vitamin A, $C_{20}H_{29}OH$, which, judging by the partition, is present in the tissues as an ester (von Euler, Karrer, Klussmann, and Morf, 1932; Wald, 1934–35).

The concentrations of both carotenoids in the pigmented layers of light and dark adapted frogs were measured with the Pulfrich photom-

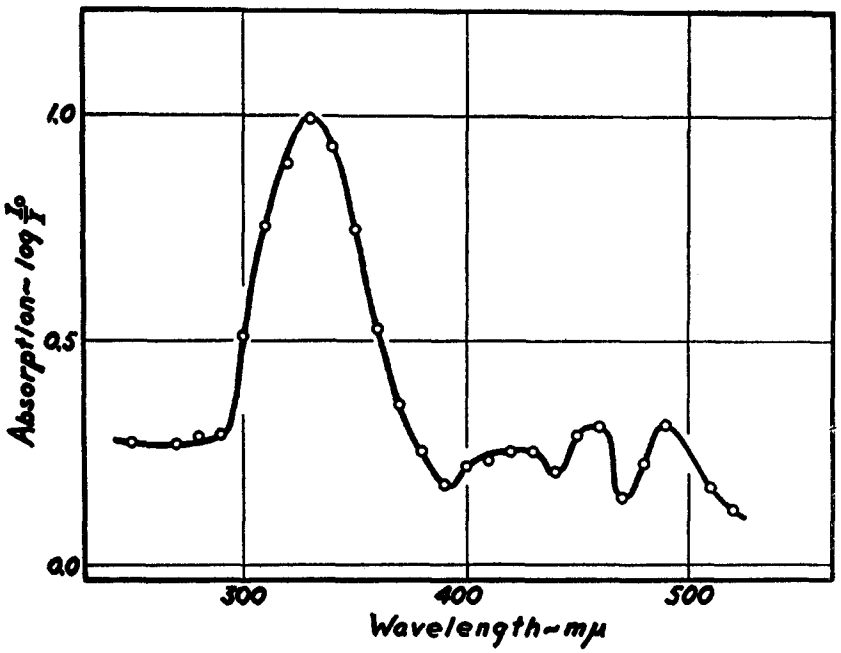

FIG. 2. Absorption spectrum of an extract of pigmented layers in chloroform (*R. pipiens*), which had been treated as in Fig. 1. The broad band at 328 mμ is due to vitamin A. Beside it is an imperfect xanthophyll spectrum, the solution having been too dilute to permit accurate measurement of this pigment.

eter (Zeiss), by methods described in the Appendix. The results are presented in Table I. The irregularity of the bull-frog (*R. catesbiana*) data is probably due to the small number of these animals used in the experiments.

In *R. pipiens* and *esculenta*, the pigmented layers of each eye contain about 4γ of vitamin A and 1γ of xanthophyll, or about 1.8 mg. of the vitamin and 0.45 mg. of xanthophyll per gram of dry tissue.

Light adaptation does not appreciably alter the vitamin A concentration, but causes a loss of 10 to 20 per cent of the xanthophyll. These measurements therefore confirm Boll's observation that the golden oil droplets of the pigment epithelium fade in animals exposed to bright light. The quantity of xanthophyll which disappears bears no simple relation to the duration of the exposure; for on 1.8.34 (Table I) the frogs had been exposed to daylight for 1 hour, while on 1.20.34 they had been left in daylight and bright electric light continuously for 3 days, without significant further change in xanthophyll content.

TABLE I

Carotenoids of the Pigmented Layers

Species	Dry weight per eye	Date	Number of eyes	Condition	Vitamin A per eye	Xanthophyll per eye
	mg.	*1933*			γ	γ
R. esculenta	2.25	Aug. 16	16	Dark adapted	4.43	0.985
		" 18	16	" "	3.92	1.00
		" 13	16	Light adapted	4.61	0.955
		" 17	16	" "	4.09	0.839
		1934				
R. pipiens	2	Jan. 11	23	Dark adapted	3.72	1.06
		" 15	22	" "	3.47	1.15
		Feb. 14	18	" "	3.65	1.17
		Jan. 8	28	Light adapted	3.85	0.802
		" 20	18	" "	3.41	0.912
R. catesbiana	3.5	" 25	4	Dark adapted	5.40	2.01
		Feb. 19	6	" "	12.8	1.74
		Jan. 22	4	Light adapted	12.7	2.67

Dark Adapted Retinas.—The retinas of dark adapted frogs may be extracted thoroughly in the dark with a homopolar organic solvent like benzine or carbon disulfide without affecting the visual purple. The extracts are colorless. When highly concentrated and tested with antimony trichloride reagent, they display the vitamin A band at 615 mμ; the concentration of the vitamin is too low to appear in the present measurements.

Such retinas may be extracted subsequently with chloroform. This solvent almost immediately decolorizes the visual purple. The extract contains a greenish yellow pigment which exhibits carotenoid

properties different from any yet reported in the literature.[8] I shall refer to this substance as retinene.

Retinene possesses no absorption bands in the visible spectrum. Its color is due to an increasing absorption from about 500 mμ into the ultraviolet. A crude extract of *R. esculenta* retinas in chloroform showed a small inflection at about 405 mμ and bands at about 310 and 280 mμ; this preparation displayed a strong basic absorption so that any of these bands that may have been due to retinene had probably been displaced.

Retinene reacts with antimony trichloride to yield a deep blue color, associated with a single sharp band at about 664 mμ.[9] Carotenoids generally yield blue to green colorations with antimony trichloride, due to spectral absorptions specific for each member of the group. All known natural carotenoids but vitamin A exhibit bands in this reaction which fall at 590 mμ or below (von Euler, Karrer, Klussmann, and Morf, 1932). The vitamin A band is at about 615 mμ. The retinene band at 664 mμ is therefore in a wholly isolated position (Fig. 3).

In the present experiments this band has been employed to identify retinene. Its optical density, determined with the Pulfrich photometer, has also been used as a measure of concentration. Principally, however, retinene concentrations were measured directly by the absorption of the substance in chloroform at 430 mμ. The ratio between this value and the absorption of the 664 mμ band in the antimony trichloride reaction is constant, showing the yellow pigment and the substance responsible for the antimony trichloride test to be identical.

Measurements of retinene concentrations in dark adapted retinas are presented in Table II. These are in relative units, equal to 10 times the optical density of the chloroform solution at 430 mμ, in a layer 1 cm. in depth.

Retinene, after extraction from the retina, is freely soluble in ben-

[8] The pigment is extracted only after shaking for some time, centrifuging, and drawing off all excess water. This process is repeated several times with the same portion of chloroform. Usually at about the third repetition the pigment suddenly appears in the chloroform.

[9] In the pocket spectroscope this band has invariably appeared at about 655 mμ, the position previously given (Wald, 1934).

zine or carbon disulfide; yet no amount of shaking with these solvents extracts it from dark adapted retinas. It is evidently bound in such retinas within some non-lipoidal complex. The conditions of its liberation by chloroform suggest that this complex is visual purple.

Light Adapted Retinas.—The retinas of frogs which have been exposed to bright daylight for one-half hour or longer are colorless, and yield with benzine or chloroform colorless extracts which contain

TABLE II

Carotenoids of the Retina

Species	Dry weight per retina	Date	Number of retinas	Condition	Vitamin A per retina	Retinene (relative units)
	mg.	*1933*			γ	
R. esculenta	3	Aug. 16	16	Dark adapted	0.00	0.66
		" 18	16	" "	0.00	0.59
		" 13	16	Light adapted	0.21	0.00
		" 17	16	" "	—	0.00
		" 21	12	Bleached and faded	1.17	0.0
		1934				
R. pipiens	3	Jan. 11	23	Dark adapted	0.00	0.43
		" 15	22	" "	0.00	0.40
		" 8	28	Light adapted	0.34	0.00
		" 20	18	" "	0.30	0.00
		Mar. 1	36	" "	0.34	0.00
		" 8	22	Bleached, 2 min.*	—	0.27
		Feb. 14	18	" , 15 min.*	—	0.16
		Mar. 16	24	" , 60 min.*	0.81	0.00
R. catesbiana	7	Jan. 25	4	Dark adapted	0.00	1.28
		" 22	4	Light adapted	1.55	0.00
		Feb. 19	6	Bleached, 45 min.*	3.61	0.00

* The times written after the notation "bleached" are periods spent at about 25°C. between bleaching and extraction.

no retinene. Such extracts do contain about 0.2–0.3γ of vitamin A per *pipiens* or *esculenta* retina (Table II). The process of light adaptation which has removed visual purple and bound retinene has produced this quantity of the free vitamin.

The mechanism of these changes is revealed in experiments with isolated retinas.

The Bleaching of Visual Purple in Isolated Retinas.—The retinas of dark adapted *R. pipiens*, when exposed to bright daylight, turn im-

mediately from the deep red visual purple color to bright orange (visual yellow). This fades slowly, and within about an hour at room temperature the retinas have become colorless. This sequence has been described in detail by Kühne (1878)[10] and Garten (1906), and has been understood to involve purely photochemical phenomena.

It can easily be demonstrated that only the first step in the process, the conversion of visual purple to yellow, is photochemical. The subsequent decolorization of visual yellow is an ordinary thermal reaction.

If dark adapted retinas are cooled to 0°C. and are exposed to bright light at this temperature, the visual purple bleaches to orange as before, but the orange color is maintained relatively unimpaired for many hours, even in bright sunlight. Upon allowing such retinas to return to room temperature, the color immediately begins to fade, and within about an hour has vanished.

Conversely, if retinas, after bleaching to orange, are placed in complete darkness at 25°C., within about an hour the visual yellow has entirely disappeared. In this case a quantity of visual purple may be regenerated, by rough estimate perhaps as much as one-third the original amount. On re-illumination such retinas assume a very faint orange color, due to the bleaching of the regenerated visual purple alone. This process may be repeated several times. Each time a fraction of the visual yellow reverts to purple, the remainder forming colorless products. Finally all of it has been converted to colorless material, which in the isolated retina never regenerates more than a trace of visual purple after several hours in darkness. The fading of visual yellow in retinas left continuously illuminated must similarly involve visual purple regeneration, though in this case the pigment is bleached as quickly as formed. Both situations end in colorless retinas which no longer have the power to spontaneously form appreciable quantities of visual purple.

At 0°C. the regeneration of visual purple from yellow is inhibited in the same way as the formation of colorless products. Both processes have the high temperature coefficients typical of thermal reactions.[11]

[10] Kühne, 1878, p. 1.

[11] Garten (1906) performed experiments similar to those reported in this

The appearance of visual yellow in retinas exposed to light depends upon the balance between the photochemical bleaching and thermal fading reactions. The velocity of the former process is principally controlled by the intensity of the light, that of the latter by the temperature. At room temperature and high light intensities, the yellow intermediate appears; at lower intensities decomposition to colorless products may keep pace with the bleaching process, so that the concentration of visual yellow remains inappreciable. At 0°C. the removal of visual yellow is so slow that it is seen to be the primary product of bleaching over a wide range of intensities.

It is concluded that light converts visual purple in the isolated retina to visual yellow, which is removed by thermal processes in two directions: (*a*) reconversion to visual purple; and (*b*) decomposition to colorless products.

Chemistry of the Bleaching and Fading Processes.—Dark adapted retinas yield retinene only after their visual purple has been destroyed with a reagent such as chloroform. After they have been exposed to light and are in the visual yellow condition, they yield their full content of retinene to benzine, carbon disulfide, or other homopolar solvents.[12] Apparently chloroform and light to this extent accomplish the same result: the disruption of visual purple and the liberation of retinene.

Visual yellow may be simply retinene. The fact that it appears greenish yellow in chloroform solution and orange in the retina does not detract from this possibility, for all of the carotenoids shift in hue from one solvent to another, and tend to assume redder tints when adsorbed. Whatever forces may hold retinene in a specific visual yellow complex must be extremely weak for so gentle a process as shaking with benzine to disrupt them. For the present, we may define retinene which cannot be extracted with benzine as bound, that which can be so extracted as free. By this criterion the effect of light upon visual purple is to liberate retinene.

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section and obtained the same results. He drew from them quite different conclusions. These will be discussed critically in a subsequent paper.

[12] At the time the preliminary report of this work was written (1934) I had used only chloroform to extract bleached retinas, and so did not know of this difference between the states of retinene in them and in dark adapted retinas.

The subsequent fate of the retinene may easily be followed. Extracts of just bleached retinas contain, as do dark adapted retinas, large quantities of retinene, but no more than a trace of vitamin A. After bleached retinas have been allowed to fade somewhat at room temperature, their extracts contain smaller quantities of retinene,

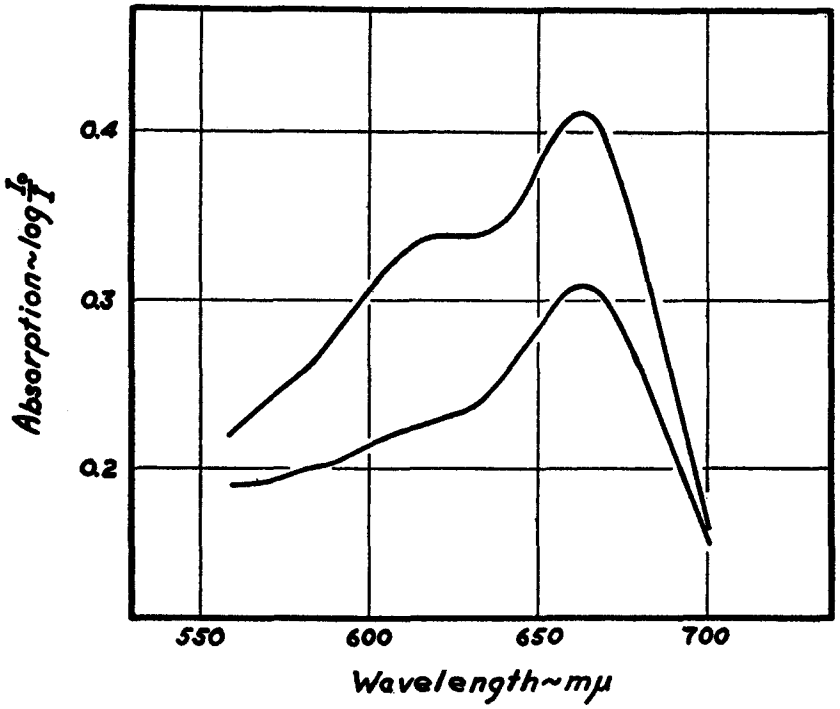

FIG. 3. Absorption spectra of the antimony trichloride reaction with benzine extracts of bleached *R. catesbiana* retinas. Lower curve: retinas extracted immediately upon bleaching. The test shows a strong retinene absorption at 664 mμ. Upper curve: retinas partly faded before extraction. The vitamin A band at 612 mμ has appeared beside the retinene absorption.

and considerable amounts of newly formed vitamin A (Fig. 3). As fading proceeds, the retinene content steadily falls and that of vitamin A reciprocally rises. Finally, in wholly faded, colorless retinas, retinene has entirely vanished and vitamin A alone is found. Measurements of retinene and vitamin A concentrations made in the

course of fading are presented in Table II. The fading process is the source of the vitamin A of light adapted retinas.

The data of Table II show that the loss of about 0.63 unit of retinene from the fading *R. esculenta* retina is accompanied by the appearance of 1.17γ of vitamin A. Similarly, about 0.42 unit of retinene in *R. pipiens* is converted into 0.81γ of vitamin A. In both cases the ratio between the units of retinene removed and vitamin A formed is the same, about 0.5. This regularity suggests that the conversion in the isolated retina proceeds stoichiometrically, a given quantity of retinene forming its molecular equivalent of vitamin A.

It is significant that the amount of vitamin A formed in the isolated retina in this way is much greater than may be found in the retina of a light adapted animal. The essential difference between these situations is that in isolated retinas vitamin A is formed irreversibly, hence only once; while in the living animal it appears as part of a continuous process in which visual purple is resynthesized and decomposed repeatedly during light adaptation. It must be assumed that some vitamin A is lost in the visual process. Ordinarily this is replaced from sources outside the retina. The large store of vitamin A in the pigment epithelium probably serves as an immediate supply. Ultimately, however, the vitamin must be derived from the diet; and the necessity of replacing vitamin A lost during the visual cycle in this manner explains the occurrence of night blindness in animals deprived of the vitamin.

DISCUSSION

The Constitution of Visual Purple.—Having concluded that visual purple contains retinene bound to some non-lipoidal material, one may inquire further into the nature of this complex.

Visual purple in bile salts solution does not diffuse through a semipermeable membrane (Ewald and Kühne, 1878).[13] It can be salted out of such solutions quantitatively with magnesium or ammonium sulfate (Kühne, 1895). These are general colloidal properties; other characteristics of visual purple suggest strongly that its colloidal residue is a protein.

In either the retina or solution, visual purple is destroyed by warm-

[13] Ewald and Kühne, 1878, Paper IV, p. 454.

ing to 60–70°C. (Ewald and Kühne, 1878).[14] The velocity of this process increases by a factor (Q_{10}) of about 7 over this range of temperatures. Kühne's data for the velocity of heat destruction in fresh retinas fit the Arrhenius expression fairly well, and reveal an activation energy of about 75,000 calories per mol. In acid or alkaline solution the process is accelerated and occurs at lower temperatures. On the other hand, desiccated retinas are comparatively resistant to heat and require several hours to turn yellow even at 100°C.

No class of phenomena now known presents exactly this combination of properties but the heat coagulation of proteins (Chick and Martin, 1910).

A second group of visual purple reactions leads to the same conclusion. Acetone, alcohol, chloroform, heavy metal chlorides, and mineral acids and alkalies all quickly decolorize visual purple either in the retina or in solution (Boll, 1877; Kühne, 1879). This series of reagents possesses only one common property: that of denaturing and coagulating proteins (Lloyd, 1926).[15]

The immediate products of visual purple decomposition by heat or chemical treatment are usually orange or yellow in color, and often these products are relatively stable to further treatment and to light. This behavior, added to its other properties, links visual purple with a well defined group of chemical substances, the carotenoid-proteins.

Combinations of carotenoids with protein are widely distributed among animals. Palmer and Eckles (1914) first discovered one in cattle serum, in which large quantities of carotene occur, bound to albumin. Vegezzi (1916) and Verne (1923) have studied a number of such complexes in the decapod Crustacea. Lwoff (1927) has de-

[14] Ewald and Kühne, 1878, Paper IV, p. 440.

[15] Not all reagents which coagulate proteins decolorize visual purple. In 4 per cent formaldehyde (Garten, 1906) or alum solutions (Kühne, 1878)[16] its color remains intact. The general coagulative changes which follow the death of the retina also do not affect it. It is significant that in these cases, though the photochemical bleaching process is unimpaired, both the fading of visual yellow and its reversion to visual purple may be completely inhibited (Ewald and Kühne, 1878);[17] (Garten, 1906).

[16] Kühne, 1878, p. 83.

[17] Ewald and Kühne, 1878, Paper IV, p. 433.

scribed them in the oocytes and ocelli of a copepod, *Idya furcata*. Kuhn and Lederer (1933) have investigated the constitution of such pigments from lobster shells and eggs, and have isolated their carotenoid component, astacin.

All of the carotenoid-proteins possess almost identical properties, significantly parallel to those of visual purple. They are non-diffusible and may be salted from aqueous solution. They yield no color to homopolar organic solvents like benzine. They are destroyed by warming, acids, alkalies, alcohol, and acetone, usually with change in color from the purple-to-green tints of the complexes to the red-to-yellow tints of the free carotenoids which may, after such treatment, be extracted easily with the usual organic solvents. This is precisely the relation between visual purple and retinene. Among these substances, visual purple is distinguished only by its extreme light sensitivity.

A direct method of testing the protein character of visual purple might be that of enzymatic digestion. Kühne's experiments of this nature have been inconclusive (1879).[18] The digestion of whole retinas with trypsin does not affect the visual purple. However, at the close of this treatment the pigment residue is always found enclosed in keratin, which is itself not attacked by trypsin and may have protected the visual purple from the enzyme. This possibility is consistent with the observation of Ayres (1878), that a trypsin preparation which did not affect visual purple in the retina decolorized it rapidly, over a yellow intermediate, when in solution. However, Ayres found his trypsin to coagulate casein also, so that its effect upon visual purple may have been analogous to that of other protein coagulants and not a true digestive action. This type of experiment requires thorough re-investigation.

All available evidence permits the assumption that visual purple is a conjugated protein, in which retinene is the prosthetic group. Any treatment which breaks this linkage or attacks the protein may discharge the color of visual purple, and allow the yellow or orange colors due to retinene to appear.

The Precursor of Visual Purple.—In order to establish the existence of a visual cycle it is necessary to show that vitamin A is the precursor

[18] Kühne, 1879, p. 267.

as well as the product of the visual purple system. The present paper has so far fulfilled only the latter requirement.

It has proved impossible to synthesize visual purple from known substances *in vitro*, or even to obtain an appreciable regeneration of the pigment in isolated light adapted (colorless) retinas. One is therefore restricted to a study of the synthetic process in the intact animal, measured either by direct retinal analysis or by the determination of visual thresholds during dark adaptation,which bear a simple relation to the retinal visual purple concentration (Hecht, 1919–20; Tansley, 1931).

Studied by both these methods, visual purple regeneration in cases of vitamin A deficiency provides the most direct evidence that this vitamin is the visual purple precursor. When mammals have been deprived of vitamin A for several weeks, the synthesis of visual purple is greatly inhibited. This has been demonstrated by direct analysis in the rat (Fridericia and Holm, 1925; Tansley, 1931) and by dark adaptation studies in rats (Holm, 1925) and in man (Treitel, 1885; Kravkov and Semenovskaja, 1934). Tansley (1933) has reported that in severe avitaminosis, rat and dog retinas may form no visual purple at all. Upon re-admission of vitamin A to the diet, severe cases of night blindness may be cured with great speed (Aykroyd, 1930). Vitamin A and its carotenoid precursors are the only dietary constituents known to produce this effect.

So far as known, no vertebrate can synthesize carotenoids *de novo*. Since vitamin A is a product of visual purple decomposition, the visual pigment in turn must be derived from a carotenoid. No carotenoids occur in the frog retina but retinene and vitamin A; in the light adapted retina, only the latter. Since such retinas regenerate visual purple *in vivo* with simultaneous loss of vitamin A, it seems evident that the latter substance has been converted into the former.

The vitamin A lost in the visual process must be replaced from outside the retina. Since the frog retina contains no blood vessels (Hyrtl, 1861), all of its metabolites must enter and leave by diffusion between it and the vascular choroid, through the pigment epithelium. One might therefore expect to find the visual purple precursor in passage through these tissues. It is consistent with our argument that the pigmented layers do contain large quantities of vitamin A.

Since the frog pigment epithelium also contains xanthophyll, it might be possible that this pigment is the visual purple precursor, as originally proposed by Boll. The visual system would then act as a mechanism converting xanthophyll to vitamin A. Kühne's criticisms of this theory, reviewed above, are still pertinent. One may add to them the following considerations: (*a*) Xanthophyll has not been found under any conditions in the frog retina. (*b*) Retinas and pigmented tissues of other animals which possess spectroscopically identical visual purple contain no xanthophyll. Such tissues do contain vitamin A (Wald, 1934–35). (*c*) The symptoms of vitamin A deficiency in mammals and birds are not relieved by administering xanthophyll in large quantities (Karrer, von Euler, and Rydbom, 1930; Kuhn, Brockmann, Scheunert, and Schieblich, 1933).

It is concluded that vitamin A is the visual purple precursor, and that the visual processes are cyclic in character.

The preceding argument has rested somewhat upon information derived from experiments upon mammals, the use of which in discussing frog vision may appear questionable. Visual purple is spectroscopically identical in frogs and mammals (Köttgen and Abelsdorff, 1896). Unpublished experiments by the author have shown that vitamin A and retinene occupy the same positions in the visual purple systems of fishes and mammals as in frogs.

The fact that neither dietary night blindness nor other symptoms of vitamin A deficiency have been demonstrated in frogs, although they are frequently kept wholly without food for months at a time, presents no special difficulty. At least 3 to 6 weeks of vitamin A deprivation are required to produce marked night blindness in mammals. Frogs are usually kept in the laboratory at about 20°C. below mammalian body temperature. Assuming all other conditions to be comparable, and the usual physiological temperature coefficient of about 2.5 to govern vitamin A depletion, it should take a frog about six times as long as a mammal, or from at least 4 to 8 months, to develop recognizable deficiency symptoms. This is beyond the usual survival period in the laboratory.

CONCLUSION

The results of the preceding discussion can be summarized in a diagram which may serve as a nucleus for further experiment (Fig. 4).

Most of the contents of this scheme have already been sufficiently treated.

The loss of vitamin A in the visual cycle is expressed in the diagram by interpolating the term, "degradation products." This is perhaps an unfortunate name for one or more substances of which nothing is known or implied but that they are colorless vitamin A derivatives. It is assumed that they eventually leave the retina by the only available route. They may constitute an important functional element of the cycle, and not merely its inefficiency.

Two processes have been discussed by which visual purple is synthesized in the retina: reversion from visual yellow (retinene), and regeneration from colorless substances, among them vitamin A.

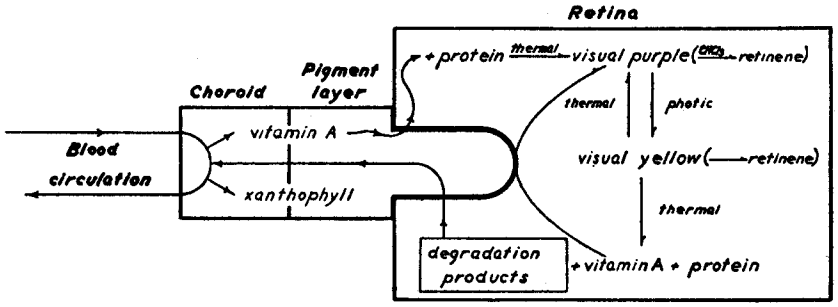

FIG. 4. Diagram of the visual purple system in the frog.

These represent two distinct bases for sensory dark adaptation, and should appear in the latter function in relative amounts which vary with the extent and period of the preceding light adaptation. This possibility is now being investigated in our laboratory.

The regeneration of visual purple from yellow appears to be a simple reversal of photolysis. The synthesis from vitamin A, however, occurs only in an eye in which the relation of the retina to the pigment epithelium has remained undisturbed (Ewald and Kühne, 1878).[19] The significance of this dependence is unknown. It is represented in the diagram by an arrow drawn tangent to the pigment epithelium.

The investigation of vitamin activity has heretofore been confined almost completely to the pathology of vitamin deficiency. The bril-

[19] Ewald and Kühne, 1878, Paper II, p. 248.

liant chemical investigations of the past few years have revealed an astonishing orthodoxy in the structure of vitamins, and have provided micro-methods for identifying and measuring them in the minute concentrations in which they occur in the tissues. It has now become possible to analyze the intimate relations between vitamins and normal physiological processes. I believe the present work to be the first of such researches to yield a positive conclusion. The function of vitamin A in the visual purple cycle is that of a simple, though special, chemical component.

SUMMARY

1. Carotenoids have been identified and their quantities measured in the eyes of several frog species. The combined pigment epithelium and choroid layer of an *R. pipiens* or *esculenta* eye contain about 1γ of xanthophyll and about 4γ of vitamin A. During light adaptation the xanthophyll content falls 10 to 20 per cent.

2. Light adapted retinas contain about 0.2–0.3 γ of vitamin A alone.

3. Dark adapted retinas contain only a trace of vitamin A. The destruction of their visual purple with chloroform liberates a hitherto undescribed carotenoid, retinene. The bleaching of visual purple to visual yellow by light also liberates retinene. Free retinene is removed from the isolated retina by two thermal processes: reversion to visual purple and decomposition to colorless products, including vitamin A. This is the source of the vitamin A of the light adapted retina.

4. Isolated retinas which have been bleached and allowed to fade completely contain several times as much vitamin A as retinas from light adapted animals. The visual purple system therefore expends vitamin A and is dependent upon the diet for its replacement.

5. Visual purple behaves as a conjugated protein in which retinene is the prosthetic group.

6. Vitamin A is the precursor of visual purple as well as the product of its decomposition. The visual processes therefore constitute a cycle.

APPENDIX

Measurement of Carotenoid Concentrations

All concentrations reported in this paper were measured with the Zeiss Pulfrich photometer.[20] This instrument measures the optical density of any desired solution for narrow bands of the spectrum, isolated by monochromatic filters. Optical density is defined as $\log I_o/I$, in which I_o is the intensity of light entering the solution, I that leaving it. For the substances and ranges of concentration with which the present work is concerned, Beer's law holds; *i.e.*, the density is directly proportional to both the concentration and the depth of the absorbing layer. This enables one to reduce the density of a solution of any depth or concentration to a standard depth of 1 cm. and volume of 1 cc., and so permits the comparison of extracts measured under a variety of conditions.

Xanthophyll is estimated directly by determining the density of the solution in chloroform at about 470 mμ, using the *S47* filter of the instrument. The density of a 1 cm. layer may be converted directly into absolute units of γ per cubic centimeter by multiplying by the factor 6.1, determined from the densities of standard solutions of crystalline xanthophyll from spinach.

Retinene is estimated similarly by the density of the chloroform solution at about 430 mμ, using filter *S43*. Ten times this value for a 1 cm. layer has been used as the relative measure of retinene concentration. The density of the antimony trichloride colorations of such solutions was also determined, using the *S61* filter. This filter, which transmits at about 610 mμ is not well adapted for retinene determinations since the retinene-antimony trichloride band maximum is at about 664 mμ. The measurements were completed within 15 seconds after mixing the reagents, since the blue color which is produced in the test begins to fade immediately. The ratio between the densities obtained by direct measurement and in the antimony trichloride reaction is constant, showing the same substance to be involved in both cases.

Vitamin A concentrations were measured by the density of the band at about 615 mμ produced with antimony trichloride, using the *S61* filter. The transmission of this filter coincides almost exactly with the absorption of the band, and so permits a very sensitive and accurate measure of vitamin A concentration. Readings completed within 15 seconds after mixing the reagents are found to obey Beer's law. The densities may be converted to γ of vitamin per cc. of the original solution by multiplying by 34. This factor was obtained by comparing the absorption of a vitamin A solution at 328 mμ with the density at 610 mμ of the same solution in the antimony trichloride reaction. Since purified vitamin A preparations in 1 per cent solution have a density at 328 mμ of about 1300 per cm. (Heilbron, Heslop, Morton, and Webster, 1932), the absolute equivalent of the antimony trichloride reaction density is easily computed. The factor offered here

[20] I wish to express my great appreciation to the firm of Carl Zeiss, Inc., for the loan of a Pulfrich photometer during my stay in Chicago.

is approximate and subject to future revision. For the present purpose relative concentrations are of much greater importance than absolute, and the absolute concentrations as given are correct at least in order of magnitude.

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