PIGMENTS OF THE RETINA

I. The Bull Frog

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The participation of certain carotenoids in the visual purple system of frogs is partly expressed in the equations (Wald, 1935–36):
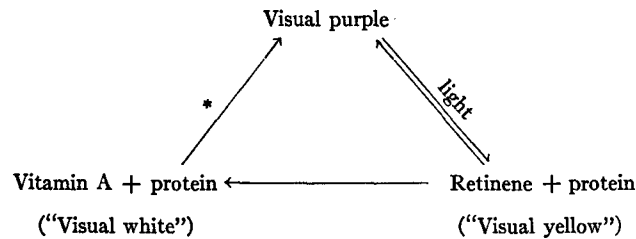
All of these reactions but the one starred occur in the isolated retina as well as in the intact eye.

In the present paper the components of this system in the bull frog, *Rana catesbiana*, are analyzed in a series of simple experiments, the results of which are presented in objective, and I believe unequivocal form.

Observations are also reported upon the distribution and properties of vitamin A, xanthophyll, and flavine in the pigmented layers of the eye.

Retinas

The retina contains varying amounts of the carotenoids vitamin A and retinene. Dissolved in chloroform in the concentrations here considered, vitamin A is colorless, retinene greenish yellow. The color of retinal extracts under various conditions thus offers a first indication of changes in these substances. With antimony trichloride reagent, both carotenoids yield blue colorations, due in the case of

781

vitamin A to an absorption band at 612–615 mμ (crude extracts), in that of retinene to one at 662–666 mμ. This reaction is used to identify both substances in the following experiments.

Dark Adapted Retinas. Visual Purple and Bound Retinene.—Dark adapted retinas may be extracted thoroughly in the dark with benzine or carbon disulfide without injury to the visual purple. The extracts
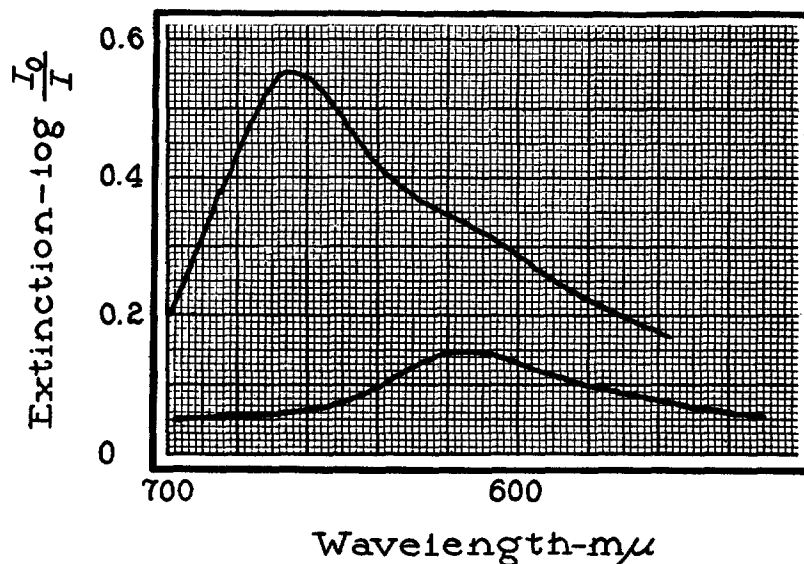

Fig. 1. The liberation of retinene from visual purple by chloroform. Spectra of antimony trichloride reactions with a benzine extract of dark adapted retinas (lower curve), and with a subsequent chloroform extract of the same retinas (upper curve). Benzine withdraws only a trace of vitamin A (615 mμ chromogen). Chloroform destroys the visual purple and extracts a large quantity of retinene (665 mμ chromogen). If the ordinates of the upper curve are multiplied by about 4, the heights of the curves represent approximate relative concentrations.

are colorless and contain a very small quantity of vitamin A alone. Subsequently, the same retinas may be extracted in the dark with chloroform, which almost immediately destroys visual purple. The chloroform extract is greenish yellow, and contains a large quantity of retinene.

Since retinene is freely soluble in benzine or carbon disulfide after it has been extracted from the retina, it must be bound in the dark

adapted tissue to some material insoluble in these reagents. Chloroform liberates it simultaneously with the destruction of visual purple.

These relations are shown in Fig. 1, which presents the results of the following experiment.[1]

Experiment.—The retinas of four dark adapted frogs were shaken thoroughly in the dark with a total of about 6 cc. benzine in three successive portions. The retinas were next extracted similarly in the dark with chloroform. The combined benzine extracts were brought into 0.3 cc. chloroform and the colorless solution used in a single antimony trichloride test. The result is shown in the lower curve of Fig. 1. The chloroform extract was greenish yellow. Tested with antimony trichloride, it yielded the upper curve of Fig. 1. Since only about ¼ the chloroform extract was used in the latter test, the ordinates of the upper curve should be multiplied by about 4 to make the heights of the curves comparable.

The Liberation of Retinene by Light.—The initial extraction of dark adapted retinas in the dark with benzine may be followed by re-extraction with benzine in bright light. Light bleaches the visual purple to a bright orange color (visual yellow), after which benzine readily extracts the total retinene. The tissue residue is colorless.

Retinene is therefore liberated by light—as by chloroform in the preceding experiment—in the destruction of visual purple. Fig. 2, obtained in the following experiment, illustrates this relation.

Experiment.—Four dark adapted retinas were shaken violently in the dark in a shaking machine for 15 minutes with a total of 6 cc. benzine in 3 successive portions. Precisely this process was repeated with the same retinas in bright daylight. Each extract was brought into 0.3 cc. chloroform. The "dark" one was colorless, the "light" one bright yellow. Both were tested with antimony trichloride. The former yielded the lower curve of Fig. 2, the latter the upper one.

Conversion of Retinene to Vitamin A in the Isolated Retina.—The retinene liberated by light is converted quantitatively to vitamin A in the isolated retina. This process is complete in about an hour at

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[1] The spectra shown in this paper were measured with a recording photoelectric spectrophotometer designed by Professor A. C. Hardy at the Massachusetts Institute of Technology (Hardy, 1935). The curves were drawn on coordinate paper by the instrument itself and have been merely mounted and reproduced. Absorptions are plotted as extinction or optical density, log (I_o/I), in which I_o is the incident and I the transmitted intensity. This quantity is directly proportional to concentration and to the depth of the absorbing layer.

25°C. It is evidenced by the fading of the bleached (visual yellow) retina to colorlessness. Fig. 3, the result of the following experiment, demonstrates this change.
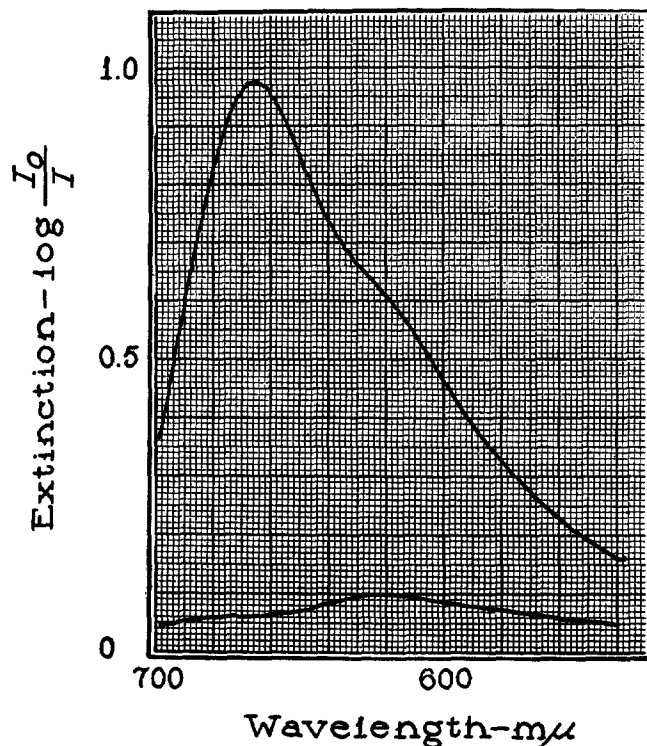

FIG. 2. The liberation of retinene from visual purple by light. Spectra of antimony trichloride reactions with benzine extracts of dark adapted retinas (lower curve),[2] and of the same retinas subsequently bleached to the visual yellow stage in bright light (upper curve). Compare with Fig. 1.

Experiment.—Right and left retinas from eight dark adapted frogs were separately prepared. One set of eight retinas was extracted in the dark with about 12 cc. chloroform in four portions. The extract was concentrated to about 1.5 cc. A sample of this, tested with antimony trichloride, yielded the upper series of curves in Fig. 3. The curves were measured consecutively on a single antimony trichloride test and follow the fading of the blue color produced in this reaction.

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[2] This curve was drawn 5 mμ too high in wavelength due to a fault in calibration which was corrected before the upper curve was recorded.
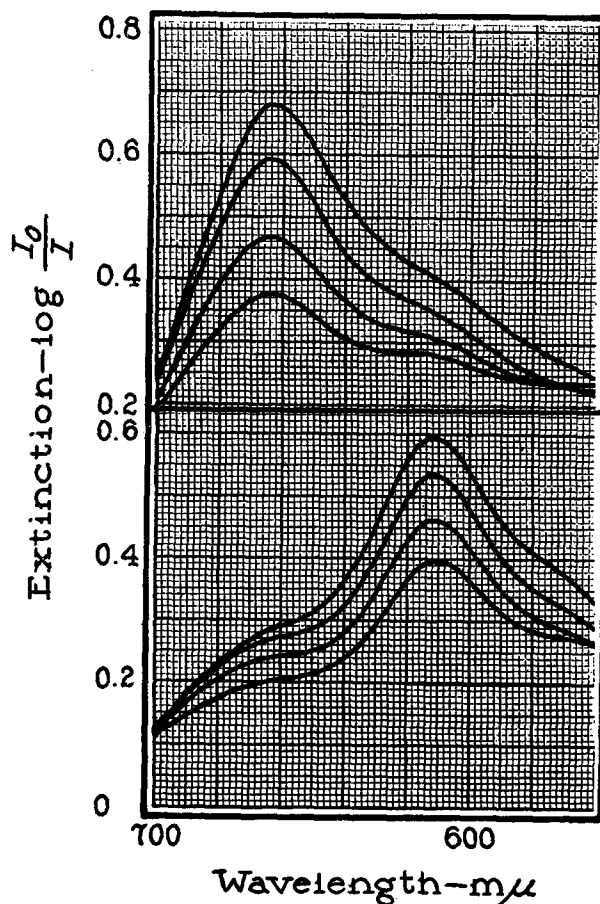
FIG. 3. The conversion of retinene to vitamin A. Spectra of antimony trichloride reactions with chloroform extracts of dark adapted retinas (upper series) and of retinas from the same animals, bleached and allowed to fade to colorlessness (lower series). Retinene (665 mμ chromogen), bound in the dark adapted retina, is liberated by light and converted during the fading process to vitamin A (612 mμ chromogen). Each series of spectra consists of successive measurements of a single antimony trichloride test, and follows for about 15 minutes the disappearance of the blue color produced in this reaction.

The second set of eight retinas from the same frogs was bleached in bright daylight and left in moderate light at 22°C. for about an hour. The retinas were then treated exactly like the former group. In the antimony trichloride test they yielded the lower series of curves of Fig. 3.

Thermal Nature of the Conversion of Retinene to Vitamin A.—The formation of vitamin A from retinene is a typical thermal reaction. It is inhibited enormously even in bright sunlight by cooling the retinas to 0°C. At room temperature it occurs in the dark with about the same speed as in the light. Fig. 4, obtained in the following experiment, illustrates the latter phenomenon.
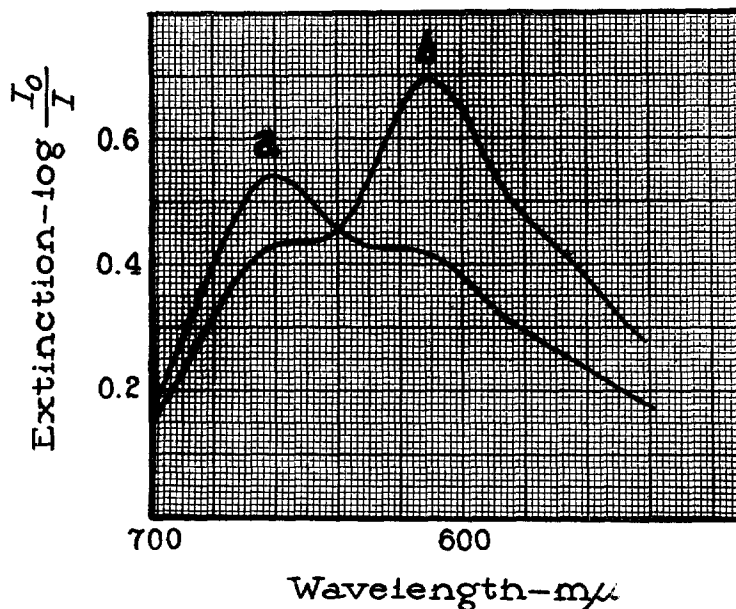

FIG. 4. Conversion of retinene to vitamin A in the dark. Spectra of antimony trichloride reactions with extracts of retinas bleached to the visual yellow condition and replaced in darkness: (a) extracted after 2 minutes in the dark; (b) extracted after 69 minutes in the dark. Compare with Fig. 3.

Garten (1906) suggested that light decomposes visual purple directly into an equimolecular mixture of visual white and visual yellow, the latter reverting quantitatively to visual purple in the dark. This view is confuted by the experiment, which shows that visual white (*i.e.*, vitamin A and other colorless substances) is formed from visual yellow by a secondary process independent of the illumination.

Experiment.—Right and left retinas of six dark adapted frogs were prepared separately. Both sets were simultaneously exposed to sunlight for 20 seconds; the visual purple was bleached almost instantly to a bright orange color. Both

sets were then placed in complete darkness at 22°C. After 2 minutes one set was extracted in the dark with chloroform. This extract yielded Curve *a* of Fig. 4. After 69 minutes in the dark, the second set of retinas was similarly extracted. It yielded Curve *b* of Fig. 4.

Light Adapted Retinas.—In the isolated retina vitamin A is the final product of the bleaching and fading reactions. In the living animal, however, the vitamin is re-synthesized to visual purple, completing
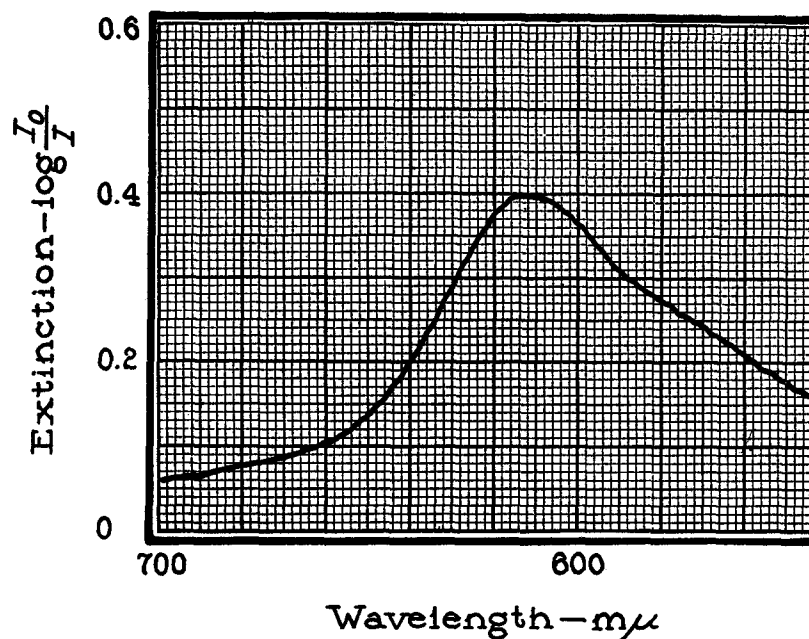

Fig. 5. Light adapted retinas. Spectrum of the antimony trichloride reaction with an extract of retinas from animals adapted to bright daylight.

the visual cycle. In the frog this process occurs even in extirpated eyes in which the relation of the retina to the pigment epithelium has not been disturbed (Ewald and Kühne, 1878).

In a frog exposed to a constant intensity of illumination the visual cycle attains a steady state, in which the concentrations of visual purple, retinene, and vitamin A remain constant, each being formed as fast as removed. At low illuminations the steady state is close to the dark adapted condition, characterized by much visual purple and

little free vitamin A and retinene. In bright light it is displaced toward the opposite end of the cycle, resulting in an accumulation of vitamin A.

In accord with this analysis the retinas of frogs adapted to bright daylight are found to be colorless, and contain vitamin A alone, in quantities very much greater than dark adapted retinas. Fig. 5 presents the result of the following typical experiment.

Experiment.—Four frogs were exposed to bright diffuse daylight for $\frac{3}{4}$-$1\frac{1}{4}$ hours. The retinas, which were colorless, were prepared in daylight. Adhering pieces of pigment epithelium were carefully picked away with fine forceps. The cleared retinas were extracted with chloroform, and the concentrated extract tested with antimony trichloride. Fig. 5 shows the result.

Concentrations: Summary.—Concentrations of vitamin A and retinene were measured by methods which have already been described (Wald, 1935–36). The amounts of these substances per retina found in groups of twenty retinas are included in the following table, which summarizes the results of the foregoing procedures.

Retinal condition	Pigmentation	Benzine extract	Chloroform extract
Dark adapted	Visual purple	Trace of vitamin A	Trace of vitamin A + 1.8 units retinene[3]
Bleached	Visual yellow	Trace of vitamin A + 1.8 units retinene	
Bleached and wholly faded	Colorless (visual white)	2.3 γ vitamin A	
Light adapted	Colorless	0.8 γ vitamin A	

As in other frog species, isolated retinas which have been bleached and allowed to fade completely contain very much more vitamin A than retinas light adapted *in vivo*. I originally interpreted this observation to indicate destruction of vitamin A in the active retina, and assumed that it accounts for the dependence of the visual purple system in some animals upon a continuous accession of vitamin A in the diet (Wald, 1935–36).

This datum may be interpreted more simply. It is sufficient to

[3] A unit of retinene is defined arbitrarily as ten times its optical density in chloroform solution at 430 mμ (Filter S43 of the Pulfrich photometer) in a layer 1 cm. in depth.

assume that in the intact eye some of the vitamin A liberated in light adaptation diffuses out of the retina into neighboring tissues. In the isolated retina, of course, this is impossible. Presumably, partition factors govern the distribution of vitamin A among the tissues. The small quantity of free vitamin found in the dark adapted retina is probably its equilibrium concentration. During light adaptation this is exceeded and some vitamin diffuses away. During dark adaptation the retina recaptures vitamin A by binding it in non-diffusible form in visual purple.

It is unnecessary therefore to assume that vitamin A is destroyed in the visual cycle. The term, "degradation products," is superfluous in the diagram of the visual purple system (Wald, 1935–36). The organism's continuous demand for vitamin A must be ascribed to a loss in processes still unidentified.

Pigmented Layers

The combined pigment epithelium and choroid layer of an eye of *R. catesbiana* contain about 2γ of xanthophyll and about 9γ of vitamin A. At least 80 per cent of these quantities is located in the pigment epithelium alone. This single layer of cells, which comprises only about ⅓ the total pigmented tissue and, which when dried weighs about 1.2 mg., therefore contains about 1.3 mg. of xanthophyll and about 6.0 mg. of vitamin A per gram dry weight.

Experiment.—Four frogs in ice water were adapted to diffuse daylight and their retinas were prepared in a room kept at 6°C. Under these conditions most of the pigment epithelium attaches very firmly to the retina and is removed from the fundus with it, leaving the choroid layer alone behind. The latter was scooped into Ringer's solution with a small spatula. The retinas were warmed to about 22°C. At this temperature the pigment epithelia adhere less closely and may be picked away from the retinas with forceps. Pigment epithelia and choroid layers were collected separately out of the Ringer in which they had been prepared by centrifuging. Each group of tissues was extracted with benzine, and the concentrations of xanthophyll and vitamin A were measured.

	Combined pigmented layers γ	Per cent in pigment epithelium	Per cent in choroid
Xanthophyll per eye	2.2	77	23
Vitamin A per eye	6.9	84	16

Xanthophyll.—Xanthophyll may be extracted from the pigmented layers with benzine. When the resulting solution is shaken with 90 per cent methanol, the pigment enters the benzine layer almost quantitatively (epiphasic). After saponification this partition is reversed, the pigment entering the methanol (hypophasic). Before saponification the pigment is strongly adsorbed from benzine by powdered aluminum oxide, though it passes readily through calcium carbonate. After saponification it is strongly adsorbed by the latter material.
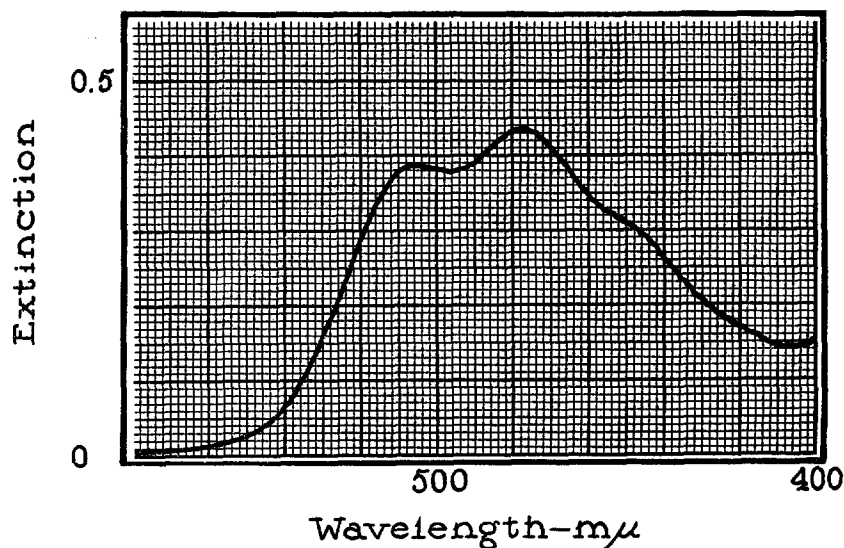

Fig. 6. Spectrum of a carbon disulfide solution of xanthophyll from pigmented tissues. This preparation was partly purified by saponification, partition between 90 per cent methanol and benzine, and adsorption on calcium carbonate.

These changes in behavior on saponification indicate that the xanthophyll occurs in the tissues as an ester.

Xanthophyll preparations from the pigmented layers deteriorate easily with age, yielding derivatives which may obscure the outcome of the experiments greatly. Portions of the pigment may turn permanently epiphasic, though changing but little in spectrum. Other portions which remain hypophasic may shift several $m\mu$ in spectrum toward shorter wavelengths. These are adsorbed in a $CaCO_3$ column

above the xanthophyll itself. Kuhn and Brockmann (1932) have reported similar changes in xanthophyll preparations from plant tissues. It was due to the presence of such impurities that the spectrum of a crude pigment layer preparation presented earlier diverged appreciably from that of crystalline xanthophyll (Wald, 1935–36).

The spectrum of free xanthophyll from pigmented tissue, purified by partition and adsorption on calcium carbonate, is shown in Fig. 6. This agrees within the errors of measurement with the spectrum of crystalline xanthophyll (lutein, $C_{40}H_{54}(OH)_2$) (Kuhn and Smakula, 1931). The spectrum shown here is somewhat more diffuse than that of the crystalline material, due partly to the fact that it was measured with an instrument of wide slit-width (5 mμ at the objective) which tended to level maxima and minima slightly.

These xanthophyll preparations, freed of vitamin A by adsorption on calcium carbonate, and concentrated in chloroform, yield a blue-green color when mixed with antimony trichloride reagent, due to an absorption band at about 585 mμ. This response also is characteristic of crystalline xanthophyll (von Euler, Karrer, Klussmann, and Morf, 1932).

The methods for treating and identifying carotenoid extracts referred to here have been described by Kuhn and Brockmann (1932) and Karrer and Schöpp (1932), and have recently been reviewed in detail by Zechmeister (1934).

Vitamin A.—When a saponified extract of pigmented layers in benzine is poured through a column of powdered $CaCO_3$, xanthophyll is quantitatively adsorbed and vitamin A emerges alone in the colorless washings. Brought into chloroform and treated with antimony trichloride reagent, it yields the sharp band at 620 mμ specific for this vitamin (Fig. 7). Crude extracts yield bands in the same reaction at 612–615 mμ. The shift to lower wavelengths is due apparently to the presence of contaminating substances, for the adsorption treatment alone moves the band to 620 mμ, even in unsaponified preparations.[4]

Like the xanthophyll, vitamin A, if partitioned between 90 per cent

[4] Fish liver oils show similar and more extreme behavior. The antimony trichloride-vitamin A band in crude oils is at 600–610 mμ. After partial purification this is shifted to 620 mμ (Heilbron, Gillam, and Morton, 1931).

methanol and benzine, is epiphasic as extracted, but hypophasic after saponification.   It appears therefore to occur in the tissues as an ester (compare Karrer, Morf, and Schöpp, 1931).   One preparation, partially purified by adsorption, exhibited the following properties:

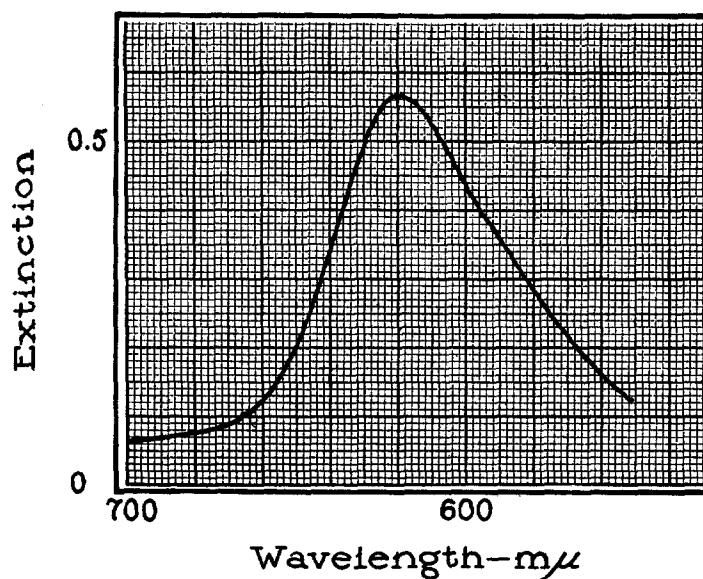|	Per cent in benzine	Per cent in methanol
Unsaponified..	98.6	1.4
Partially saponified in 5 per cent ethanol-KOH for 25 minutes at 40°C...	59.9	40.1

FIG. 7.   Spectrum of the antimony trichloride reaction with vitamin A from pigmented tissues, freed from xanthophyll and other impurities by adsorption methods.

Flavine.—After pigmented tissue has been extracted exhaustively with benzine it may be re-extracted with 80 per cent aqueous acetone. The extract contains a lemon-yellow pigment which fluoresces strongly green.   On adding acids or alkalies the fluorescence vanishes and returns on neutralizing.   If a drop of dilute sodium hydrosulfite solution is added to the extract made just alkaline to litmus, the pigment is decolorized.   On shaking with air it regains its yellow color.   This

reaction may be repeated indefinitely. The properties identify the pigment as a flavine (lyochrome) (Kuhn, György, and Wagner-Jauregg, 1933 a, b). Von Euler and Adler (1934 a, b) have reported the presence of flavines in the eye tissues of a number of fishes and mammals.

The absorption spectrum of crude flavine from bull frog pigmented layers is shown in Fig. 8. This agrees fairly well with the spectrum of similar extracts from fish pigmented layers (von Euler and Adler, 1934a) and with that of crystalline lactoflavine (Kuhn, György, and Wagner-Jauregg, 1933 b). An incomplete extract of pigmented tissues from 28 eyes contained about 1.3γ of flavine per eye. The measurement was
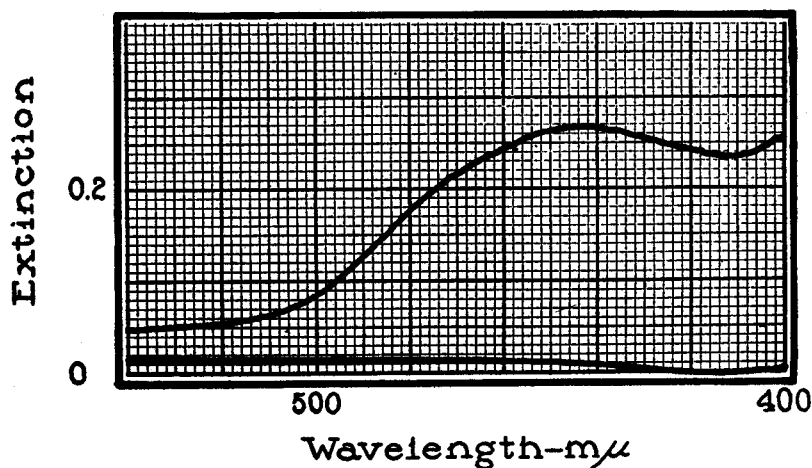

FIG. 8. Spectrum of a water solution of crude flavine from pigmented tissues. The lower curve is the reference spectrum of distilled water alone.

performed in the Pulfrich photometer, and is based on the fact that in this instrument 100γ of crystalline lactoflavine per cubic centimeter of aqueous solution, measured with the S47 filter in a layer 1 cm. in depth, has an optical density of 3.2 ± 0.15 (Kuhn, Reinemund, Weygand, and Ströbele, 1935).

Lactoflavine, obtained from whey, liver, eggs, and grass has been shown to be identical with vitamin B_2 or G (Kuhn, Rudy, and Wagner-Jauregg, 1933; von Euler, Karrer, Adler, and Malmberg, 1934). Flavines combined with protein appear to form an extensive group of closely related redox enzymes, of which the *gelbe Ferment* of War-

burg and Christian (1932) alone has been isolated (Theorell, 1935). No attempt has been made in the present work to determine whether the retinal flavine occurs in free or bound form in the tissues. In the fish eye von Euler and Adler found it to be almost entirely free, and restricted to the pigment epithelium. Its function in the eye is unknown. Von Euler and Adler have suggested that it may behave as a photosensitizer in the retina. It might well play some part in the extraordinarily powerful respiratory and fermentative system of this tissue.

Blue-Fluorescent Substances.—Von Euler and Adler (1934 *b*) have described certain unidentified substances in fish pigmented tissues which possess very strong blue fluorescence. Water extracts of bull frog pigmented layers contain similar substances. No attempt has been made in the present work to identify them or measure their quantities.

SUMMARY

1. The interrelations of visual purple, retinene, and vitamin A in the bull frog retina are analyzed in simple experiments, the results of which are presented in a series of automatically recorded spectra.

2. Observations are reported upon the distributions, properties, and concentrations of xanthophyll, vitamin A, and flavine in the pigmented tissues of the eye.

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