VITAMIN A IN EYE TISSUES

By GEORGE WALD*

(From the Kaiser Wilhelm-Institut für Zellphysiologie, Berlin-Dahlem, Germany, and the Chemical Institute of the University of Zürich, Zürich, Switzerland)

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Animals deprived of vitamin A become night blind: after exposure to bright light, they fail to see at intensities which still readily stimulate the normal eye. This is due to a delay in the dark adaptation processes which normally replace the visual purple bleached by light.[1] Fridericia and Holm (1925) and Tansley (1931) have demonstrated directly that in rats suffering from avitaminosis A visual purple is synthesized more slowly than in normal animals. In extreme cases none may be formed in the retina at all (Tansley, 1933).

The dependence of visual purple formation upon vitamin A is most direct. About 3 weeks of vitamin A deprivation may result in severe night blindness in man (Aykroyd, 1930) and in the rat (Holm, 1925) before any other deficiency symptom can be recognized. In both animals a small quantity of cod liver oil may cure the disorder within a day.

* National Research Council Fellow in Biology; now at the Biological Laboratories, Harvard University. A preliminary report of this work appeared in *Nature* (1933). The research was begun in the Kaiser Wilhelm-Institut für Zellphysiologie, Berlin-Dahlem, and I am much indebted to Professor Otto Warburg for the facilities placed at my disposal there. I wish also to thank Dr. Negelein and Mr. Haas of that laboratory, particularly for measuring the spectra shown in Fig. 2. The work was completed at the Chemical Institute of the University of Zürich. I am happy to thank its director, Professor Paul Karrer, for many kindnesses and much patient advice and supervision.

I am particularly indebted to my wife, Frances Kingsley Wald, for having prepared most of the 6000 pig and 5000 cattle retinas used in these experiments.

[1] Blegvad's paper upon the relation between vitamin A and various eye abnormalities (1924) is classic in this field. A more detailed treatment of night blindness may be found in the pioneer work of Kubli (1887) and a recent review by Dieter (1931).

The intimacy of this relation suggests that vitamin A is involved directly in visual purple synthesis, and so should occur in the eye tissues. Several investigators have attempted to identify it there.

Holm (1929) has shown that fresh calf retinas added to an otherwise vitamin A-free diet may cure rats suffering from avitaminosis A. Yudkin, Kriss, and Smith (1931) have obtained similar results with dried pig retinas. Such curative activity is not limited to vitamin A, however, but is shared to a high degree by kryptoxanthin (Kuhn and Grundmann, 1933) and the three isomeric forms of carotene (Kuhn and Brockmann, 1933) among the natural carotenoids. Yudkin, Kriss, and Smith also found that ether extracts of the pig retina yield a blue color with arsenic trichloride. This reaction again is not specific, but is given by the carotenoid pigments generally. The presence of vitamin A in the eye tissues is therefore still to be demonstrated.

The present experiments show that considerable quantities of vitamin A occur in the retinas and the combined pigment epithelia and choroid layers of frogs, sheep, pigs, and cattle.

Experiments

Eye tissues were washed twice in saline, and then repeatedly in distilled water until no hemoglobin remained in the washings. About 15 gm. fresh weight of tissue were mixed with 10 cc. of 95 per cent alcohol and 1 cc. of 10 N aqueous potassium hydroxide, and kept at 75°C. for 20 minutes to saponify. The tissues were completely disintegrated by this process. The mixture was cooled, diluted with water, and refluxed with benzine at 60°C. for 20 minutes. The benzine layer was drained off, and the aqueous layer extracted cold a second time with benzine. The combined benzine extracts were washed thoroughly with distilled water, then dried overnight with anhydrous sodium sulfate. The benzine was distilled off under reduced pressure, and the yellow, oily residue taken up in a few cubic centimeters of dry chloroform and stored under argon at 0°C. This solution was employed in the following tests.

Antimony Trichloride Reaction.—Extracts of various eye tissues, mixed with a saturated chloroform solution of antimony trichloride, always yielded the blue color characteristic of the carotenoids (Carr and Price, 1926; von Euler, Hellström, and Rydbom, 1929). This faded almost completely within several minutes, the solution finally turning a permanent red.

The initial blue color is due to selective absorption of the longer wave

lengths of the visible spectrum, specific for each carotenoid. In this test vitamin A may be distinguished easily from all the other known members of the group through its absorption band at about 620 mμ, since no other natural carotenoid yields bands above 590 mμ (von Euler, Karrer, Klussmann, and Morf, 1932). The vitamin A absorption at 620 mμ falls rapidly after the antimony trichloride has been added, while in impure preparations secondary bands appear at shorter wave lengths, which cause a red color in later stages of the reaction (Wokes, 1928).

Retinal and pigment layer extracts from frogs, sheep, pigs, and cattle display all of these characteristics. The absorption band at about 620 mμ is always sharply defined. A very faint additional band at about 580 mμ is sometimes observed in more concentrated preparations. This may be due to some hepaxanthin, which always accompanies vitamin A in fish liver oils (van Eekelen, Emmerie, Julius, and Wolff, 1932; von Euler, Karrer, and Zubrys, 1934); or to an oxidation product of the vitamin (Brockmann and Tecklenburg, 1933). Neither substance appears to possess vitamin activity. The permanent red color which succeeds the initial blue coincides with the appearance of a series of bands, the strongest at about 500 mμ, and others at about 530 and 560 mμ. These are of no special interest, since a number of organic substances yield delayed red colorations with antimony trichloride (Levine and Richman, 1933).

The spectrogram of the antimony trichloride reaction with an extract of cattle retinas, shown in Fig. 1, illustrates these properties. For comparison, a similar photograph of this reaction with halibut liver oil was taken upon the same plate. The two spectra are obviously identical.

Absorption Spectrum.—Chloroform solutions of vitamin A possess a single broad absorption band in the near ultraviolet, the maximum of which is at 328 mμ. The remaining natural carotenoids—except hepaxanthin, with a band at 270 mμ—all have absorption bands in the visible spectrum, lying between 400 and 500 mμ (von Euler, Karrer, Klussmann, and Morf, 1932). The 328 mμ band thus distinguishes vitamin A clearly from the other carotenoids. Absorption spectra of the extracts in chloroform of sheep and ox retinas and pigmented layers, and of pig retinas are shown in Fig. 2. The extinction coeffi-

cients for each preparation have been multiplied by a factor, to give all of the maxima the same height on the ordinates. This is tantamount to bringing all the preparations to an equivalent concentration, since concentration is directly proportional to the extinction coefficient.

The specific vitamin A absorption at 328 mμ dominates all of these spectra. The curves rise without inflection from 500 mμ on, indicating

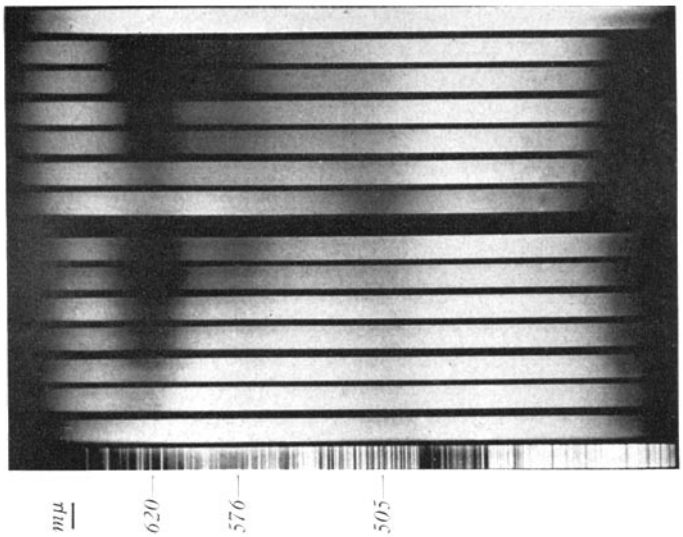

FIG. 1. Spectrograms of the antimony trichloride reaction with an ox retinal oil (above) and with halibut liver oil (below). Each series consists of 5 second exposures made at 5, 20, 32, 60, 120, and 240 seconds (reading from the top down) after mixing the reagents. Both show the 620 and 580 mμ bands fading as the reaction proceeds, and the retinal series also the growth of a secondary absorption at about 505 mμ. The first and next to last spectra are of the antimony trichloride solution alone; the last is that of the iron arc.

that no other known carotenoids, excepting possibly hepaxanthin, are present in the extracts from these tissues.

Feeding Experiments.—These were performed at the Pharmacological Institute of Hoffmann-La Roche, Basel, Switzerland, using an oil from ox retinas.

2,000 ox retinas had been collected in 95 per cent alcohol over a period of 3 weeks. The solid material was centrifuged out, mixed with about 800 gm. anhydrous sodium sulfate to dehydrate, and was extracted repeatedly with benzine.

The alcoholic mother liquor was concentrated and similarly extracted. The combined extracts were brought into methanol, and large quantities of sterols were frozen out in solid carbon dioxide-acetone mixture. The residue from the first freezing was redissolved in methanol and refrozen. The filtrates from both freezings were kept separate. They were brought into benzine, washed, and dried over sodium sulfate. After distilling away the benzine, the oily residues were sealed in high vacuum. Two sterol-free preparations resulted, with cod liver oil (c.l.o.) values of 28 and 20. The total vitamin A content in all fractions, measured with a Lovibond tintometer, was 2.12 mg.[2]

Rats had been kept on a vitamin A-free diet until deficiency symptoms appeared: loss of weight, obvious reddening of the conjunctiva, avoidance of light, and in some cases turbidity of the cornea (xerophthalmia). They were then fed small quantities of the retinal oil daily. Their weights were measured in 3 day intervals.

The first preparation (28 c.l.o. units) was administered in daily doses of 1.5 mg. After 14 days the xerophthalmia had entirely disappeared, and the animals were again growing. Smaller dosages were not tried due to lack of material.

The second preparation (20 c.l.o. units) cured the avitaminosis completely in daily doses of 1 mg.; 0.3 mg. was found to be insufficient. Intermediate dosages were not attempted. The purest vitamin A preparations test at about 10,000 c.l.o. units (Karrer and Morf, 1933). It follows that this retinal oil contained about 0.2 per cent, and the minimal curative dose between 0.6γ and 2γ, of pure vitamin A.

Karrer's sturgeon liver preparations maintain growth in normal animals in daily doses of 0.3γ. From 0.5 to 2γ per day of other fish

[2] The units are those recommended in the Report of the Cod-liver Oil Colour Test Sub-committee, Pharmacopoeia Commission Reports, London, March, 1931 (cited from Karrer, von Euler, and Schöpp, 1932). The c.l.o. unit is defined in terms of the blue value of the antimony trichloride coloration, measured in a Lovibond tintometer, by the formula

$$\text{C.L.O. units} = \frac{20 \times \text{blue value}}{\text{mg. oil per cc. chloroform solution}}$$

Since highly purified vitamin A preparations have a c.l.o. value of about 10,000 (Karrer, von Euler, and Schöpp, 1932; Karrer and Morf, 1933), the concentration of vitamin A in an oil may be computed in absolute units from the same formula. Substituting 10,000 for the left hand expression, the denominator of the right hand one becomes milligrams of vitamin A per cubic centimeter of solution.

liver oils having comparable c.l.o. values are needed to produce the same effect (Karrer, von Euler, and Schöpp, 1932). The requirement for simple maintenance appears to be less than for curing avitaminosis. The retinal and the purified fish liver preparations are therefore in good quantitative agreement.

Concentrations.—Two non-biological methods are in standard use for measuring vitamin A concentrations. The first depends upon the blue color produced with antimony trichloride and measured as already described in the Lovibond tintometer. The second method uses the extinction coefficient at 328 mμ as the direct measure of concentration. A 1 per cent chloroform solution of the purest vitamin A preparations

TABLE I

Quantities of Vitamin A in Eye Tissues

Animal	Tissue	Dry weight per tissue	Method	γ Vitamin A per tissue	γ Vitamin A per gm. dry tissue
		mg.			
Ox	Retina	51	Absorption, 328 mμ	1.05	20.6
	Retina		Lovibond	1.06	20.7
	Retina		Lovibond	1.02	20.0
Sheep	Retina	26	Absorption, 328 mμ	0.65	25.0
	Pigmented layers	32	Absorption, 328 mμ	0.75	23.5
Pig	Retina	21	Absorption, 328 mμ	0.51	24.2
	Retina		Lovibond	0.36	17.4
Frog (*R. esculenta*)	Retina	3.0	Pulfrich	1.25	415
	Pigmented layers	2.25	Pulfrich	4.24	1890

has an extinction coefficient at 328 mμ of about 1350, when in a layer 1 cm. deep (Heilbron, Heslop, Morton, and Webster, 1932). Since the extinction coefficient is directly proportional to both the concentration and the depth of the absorbing layer, the concentration of any unknown vitamin A solution may be computed from these figures.

Measurements of both these types are presented in Table I. At the time the frog extracts were prepared no Lovibond tintometer was available; in this case the absorption of the 620 mμ band was measured in a Pulfrich photometer (Zeiss), and then reduced to Lovibond units (van Eekelen, Emmerie, Julius, and Wolff, 1932).

Two features of the data are of interest. The first is the constancy

of the proportion by weight of vitamin A in the mammalian tissues. The quantity of vitamin A per gram dry weight of retina in the various species is in all cases about 22γ.

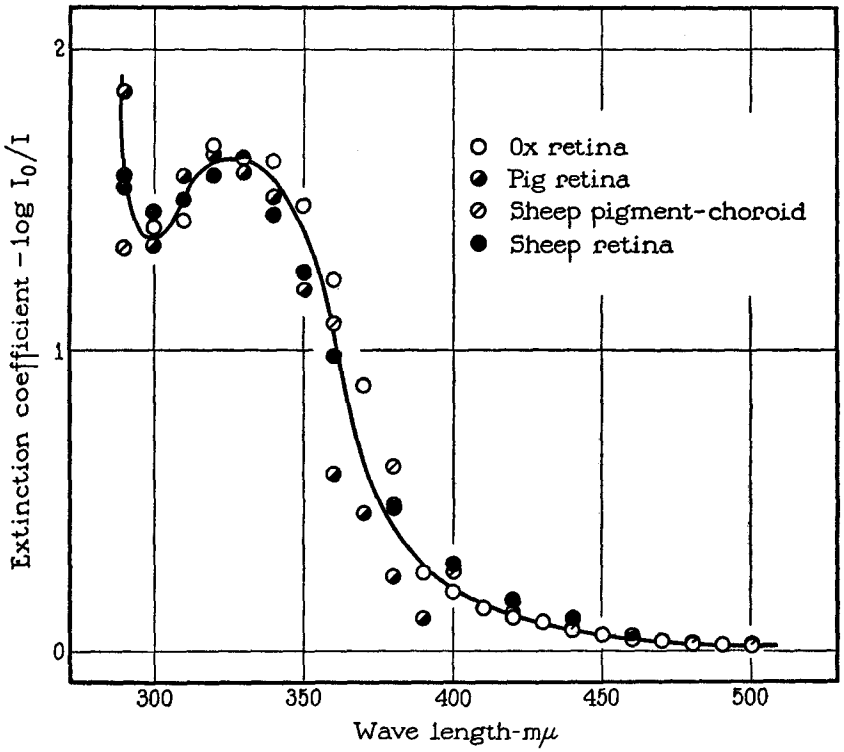

FIG. 2. Absorption spectra of eye tissue extracts in chloroform. The absorption is plotted as the extinction coefficient, log I_o/I, in which I_o is the intensity of monochromatic light entering the test solution, I that leaving it. All of the curves possess the vitamin A maximum at 328 mμ. The rise at shorter wave lengths is due to an unknown component. These spectra resemble throughout those of cod liver oils (Drummond and Morton, 1929).

The frog retina weighs about 1/17 as much as an ox retina, yet contains considerably more vitamin A. The frog pigmented layers exceed even this high concentration by about 4.5 times, and are among the very richest animal sources of the vitamin.

It is of some interest to compare these figures with the previous data

obtained by feeding rats with whole retinas. Holm (1929) found that 50 mg. of fresh calf retina displayed definite anti-xerophthalmic effects, while 750 mg. also restored optimal growth. Holm states the dry weight of these tissues to be about 12 per cent of the fresh weight. Computation of these quantities on the basis of 22γ vitamin A per gram of dry tissue yields the values 0.13γ vitamin A for a positive effect and 1.98γ for complete cure, which are consistent with the results of the present experiments.

Yudkin, Kriss, and Smith (1931) found 30 to 50 mg. per day of dried pig retina sufficient to cure rats suffering from avitaminosis A; 20 mg. per day maintained growth in normal animals, though inadequate for cures. Computed in the same way these amounts reduce to daily doses of 0.66 to 1.1γ vitamin A for curing diseased rats and 0.44γ for maintaining normal animals, figures which agree well with those obtained in the present work and occurring elsewhere in the literature.

This correspondence of the results obtained with whole retinas and with concentrates is an assurance that the vitamin A content of the retina accounts completely for its anti-xerophthalmic and growth-stimulating activity.

Retinal vitamin A concentrations might be expected to vary with the state of nutrition. I can say little concerning this matter as yet. Animals obviously suffering from lack of the vitamin have not been used in any of these experiments. The three cattle measurements were made in January, February, and June of 1933; the change from winter to summer feeding seems to have had no effect upon the retinal vitamin A.

An experiment with frog tissues is perhaps pertinent to this problem. The frogs (*R. esculenta*) were winter animals. They had been brought to the laboratory in November, and kept there at room temperature without food for about 3 months. Extracts were prepared of the combined retinas and pigmented layers and the livers of five of these animals. The liver extract was bright yellow; it gave a strong antimony trichloride reaction, showing the vitamin A band at 620 mμ and a very faint one at about 590 mμ.

The extracts from both organs were diluted so that the initial colorations with antimony trichloride were of equal intensity. From the dry weight of tissue used in each case and the final volumes of the solutions the relative vitamin A could be computed. The proportion

by dry weight of vitamin A in the eye tissues was found to be slightly more than 35 times that in the liver.

DISCUSSION

The specific activities of vitamins are still so mysterious that their functions are at present usually referred vaguely to the whole organism. The presence of vitamin A in high concentrations at the site of its most sensitive deficiency symptom—night blindness—implies some more detailed relationship. This association does not seem to be merely fortuitous, for in an exhaustive survey of the tissues of the rat, Moore (1931) has shown this vitamin to be virtually absent from all other organs but the liver.

Since the present experiments were first reported (1933), von Euler and Adler, at Stockholm (1934), have confirmed the presence of vitamin A in cattle retinas, but state that these and pigment layers also contain a yellow material which they believe to be carotene.

I have found no trace of carotene in any mammalian eye tissue, including retinas and pigmented layers from German, Swiss, and American cattle, examined during various seasons. Some yellow substance is present in the extracts from these tissues, but in no case have they exhibited spectroscopically the bands at 466 and 497 mμ characteristic of carotene, nor the band at 590 mμ which this substance yields with antimony trichloride (von Euler, Karrer, Klussmann, and Morf, 1932). The spectra reproduced in the present paper objectively confirm this observation.

The concentrations of the yellow pigment, if estimated as carotene, were found by von Euler and Adler to be about 5γ per retina and 12γ per pigment layer. Carotene has been shown to be about as potent biologically as an equal weight of vitamin A (Moore, 1933). Were these observations correct, therefore, cattle pigment layers should exhibit about twice the vitamin A activity of the retinas, and the latter tissues about six times the vitamin A potency actually realized.

SUMMARY

1. Vitamin A has been found in the retinas and the combined pigment epithelia and choroid layers of frogs, pigs, sheep, and cattle. The vitamin was identified by (*a*) its specific absorption at 328 mμ;

(*b*) the blue color yielded with antimony trichloride, associated with an absorption band at about 620 mμ; (*c*) anti-xerophthalmic and growth-promoting activity; and (*d*) quantitative relationships among the results of these three types of observation.

2. The mammalian retinas contain about 22γ, the frog retinas about 400γ, and the frog pigmented layers almost 2 mg. of vitamin A per gram of dry tissue.

3. With the possible exception of hepaxanthin, no other carotenoids were found in the mammalian tissues.

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