THE REPLACEMENT OF VITAMIN A₁ BY VITAMIN A₂ IN THE RETINA OF THE RAT

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(Received for publication, February 4, 1946)

It is now well established that vision in dim light is due to the photochemical decomposition of "visual purple," a pigment present in the rods of the retina (1). Visual purple is believed to be a conjugated protein in which vitamin A or one of its derivatives is a prosthetic group (2). As might be expected, the spectral sensitivity curve of the eye parallels the absorption spectrum of visual purple with a maximum at 500 m\(\mu\), except for a slight shift toward the red due mainly to light absorption of the intraocular media.

In 1937, Wald (3) found that certain fresh water fish have a visual purple system which differs from that found in man and most other animals. The absorption of this pigment occurs at about 522 m\(\mu\), and the entire absorption spectrum is shifted somewhat toward longer wave-lengths. It is reasonable to assume that in dim light these fish are comparatively more sensitive to red light and less sensitive to blue than animals with the normal type of visual purple. To avoid confusion, the visual purple of humans and salt water fish was named rhodopsin, while the visual purple of fresh water fish was named porphyropsin.

At about this same time, other investigators (4, 5) found that the livers of these fresh water fish contained, instead of vitamin A, a closely related substance having an absorption maximum in the ultraviolet at about 350 m\(\mu\) instead of 328 m\(\mu\) and that the antimony trichloride reaction product had an absorption maximum at 693 m\(\mu\) instead of 620 m\(\mu\). This material was named vitamin A₂, and shortly thereafter Wald (6) showed this substance to be the prosthetic group in porphyropsin, as vitamin A is in rhodopsin. It has subsequently been found (7, 8) that vitamin A₂ has biological activity for rats and occasionally occurs in small amounts in the livers of animals that eat fresh water fish (9).

It would be of considerable interest to find out whether the spectral

* Communication No. 88 from the Laboratories of Distillation Products, Inc. This work was financed in part by the War Research Committee of the Rochester Chamber of Commerce and in part by the Institute of Optics under the supervision of Dr. Brian O'Brien.
response curve of the human retina can be shifted toward the red region by
the substitution of porphyropsin for rhodopsin in the retina. As the initial
step in such a study, we investigated the possible replacement of vitamin
A<sub>1</sub> by vitamin A<sub>2</sub> in the retina, liver, and blood of the albino rat.

**Procedure**

132 male and female rats were placed at weaning (24 days old) in
individual cages and given the “vitamin A test diet” (10). After 9 weeks on
this diet, the usual vitamin A deficiency symptoms developed and the
colony was divided into two groups. One group of 63 vitamin A-deficient
rats was examined immediately; a group of 76 normal rats was examined
simultaneously for comparison. The remainder of the colony was placed
on a daily supplementation of 100 “units” of vitamin A<sub>2</sub> given orally by
dropper in Mazola. The vitamin A<sub>2</sub> was extracted from the livers, pyloric
ceca, gastrointestinal tracts, and body fat of wall-eyed pike (*Stizostedion
vitreum*). These extracts all had an ultraviolet absorption maximum at
352 m<sub>μ</sub>, with a subsidiary peak at 286 m<sub>μ</sub>. The antimony trichloride
products all showed a single absorption maximum at 695 m<sub>μ</sub>.

Groups of six to eight rats were taken at intervals of 3, 6, and 12 weeks
and examined to see what changes had occurred in the vitamin A<sub>1</sub> and A<sub>2</sub>
levels in the retina, blood, and liver. After 12 weeks on vitamin A<sub>2</sub> feeding,
a group of forty-eight rats was examined.

At the time of sacrifice, the rats were placed overnight in a dark room
and killed by decapitation. A minimum of illumination was furnished by
a Kodak Series No. 2 Safelite. The blood was collected and allowed to clot
and the serum taken. A representative number of livers were removed,
and the pooled sample weighed and placed under 95 per cent ethyl alcohol.
The eyes were removed promptly and placed in normal saline solution.
They were transferred to a 4 per cent alum solution for a period of about 2
hours. The lenses were removed, and the retinas dissected out and placed
in a phosphate buffer solution (pH 6.8), centrifuged, and washed with the
same solution repeatedly. The washed retinas were ground with sand
under 4 per cent sodium glycocholate solution and the volume brought up
to about 15 ml. After standing for an hour, the mixture was centrifuged
and the supernatant liquid poured off.

1 Since there is no standard unit of vitamin A<sub>2</sub>, we have adopted, pro tem, the
expedient of employing an arbitrary physicochemical or spectral unit. This is deter-
mined by using the same conversion factors as are used in this laboratory for calcu-
lating units of vitamin A<sub>1</sub> from its extinction coefficient at 328 m<sub>μ</sub> in the ultraviolet
or the extinction coefficient at 620 m<sub>μ</sub> for its antimony trichloride product. Thus the
potencies in units of vitamin A<sub>2</sub> were calculated by multiplying the extinction coeffi-
cient at 352 m<sub>μ</sub> by 2000 and, for the antimony trichloride product, by multiplying the
extinction coefficient at 695 m<sub>μ</sub> by 750.
The transmission spectra of the retinal extracts were determined on a Hardy spectrophotometer against a blank sample of the 4 per cent sodium glycocholate solution. The small amount of light needed to give the transmission spectra produced no bleaching of the visual purple. Subsequently, the retinal extracts were exposed to room light until no further fading occurred at 500 mp, and curves for the bleached samples were obtained. Since these extracts all had considerable general absorption in the 400 to 500 mp region, the curve of the bleached sample was subtracted from the original in order to obtain a better curve of the visual purple itself. It must be noted, however, that this procedure would yield a true spectrum of the visual purple only if it is assumed that no other products absorbing in the same region are produced at this time. Since it is known (11) that some such products are formed, the curve obtained by subtraction is not a true curve of the visual purple. However, for the purposes of this experiment, the difference is not significant.

Retinene was extracted with benzene from the bleached visual purple solutions, and its antimony trichloride spectrum recorded.

The blood sera and the livers were extracted in the usual manner and spectra of the antimony trichloride colors obtained.

**Results**

*Amount and Character of Visual Purple*—As can be seen from Fig. 1, the normal rats had a visual purple curve with a maximum at 500 mp, showing that rhodopsin was present.2 The vitamin A-deficient rats examined at the same time also showed the presence of rhodopsin but in a much smaller amount. In contrast, the large group of rats which had been on vitamin A2 supplementation for 12 weeks gave a visual purple curve with an absorption maximum at 520 mp. Since the absorption maximum of porphyropsin occurs at 522 ± 2 mp, this shift of the spectrum indicated that the visual purple of the vitamin A2-fed rats had been changed to porphyropsin to the extent of about 80 per cent or more.

The primary question with which this investigation is concerned is whether or not an animal normally utilizing only vitamin A1 in its retinal pigment can produce the typical vitamin A2-containing visual purple. Fig. 1 gives a clear cut answer to this question. On prolonged feeding of vitamin A2 the rat has almost entirely replaced vitamin A1 by vitamin A2 in the visual purple of its retina.

*Retinene*—The bleached visual purple extracts were extracted with

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2 The retinas from thirty-three frogs were examined to test our experimental procedures. An excellent rhodopsin curve was obtained. The frog retinas were found to contain about twice as much visual purple as those of the normal albino rat. The livers contained 165 units per gm. of vitamin A1.
benzene and examined for retinene, which gives a characteristic antimony trichloride blue color with a maximum at 664 \(\text{m} \mu\) (2). Definite evidence of retinene was found in the extract from the visual purple of normal rats. No retinine could be detected in the benzene extract from the bleached visual purple of rats which had been fed vitamin \(A_t\) for 12 weeks. However, in the latter case, the presence of retinene was not expected, since, according to Wald (6), bleached porphyropsin yields a different substance, retinenez. This compound gives an antimony trichloride product having an absorption maximum at 706 \(\text{m} \mu\). Unfortunately, this wave-length was beyond the limit of the spectrophotometer used.

**Vitamin \(A_1\) in Retina**—The amount of vitamin \(A_1\) in the bleached retinas of two small groups of normal rats was found to be 0.64 and 0.87 units per rat. A comparative measurement on the retinas of a small group of vitamin \(A_1\)-deficient rats showed only one-fourth to one-third as much vitamin \(A_1\) (0.23 unit per rat) as is normally present. After 3 weeks of feeding 100 units of vitamin \(A_t\) daily, the retinas of a small group showed 0.83 unit of vitamin \(A_t\) per rat. No evidence of vitamin \(A_t\) was observed. After 6 weeks of vitamin \(A_t\) supplementation, the retinas from another small group showed 0.75 unit of vitamin \(A_t\) per rat, with the appearance of just a trace of vitamin \(A_t\). It appears that the retinas pick up vitamin \(A_t\) extremely slowly and tend to maintain normal levels of vitamin \(A_1\). Since all of the available retinas were needed for the visual purple estimation on the rats fed vitamin \(A_t\) for 12 weeks, no vitamin \(A\) determinations were made on the bleached retinas of these animals.

![Absorption spectra of the visual purple extracts from normal rats (Curve a), vitamin \(A\)-deficient rats (Curve b), and previously depleted rats fed a supplement of 100 units daily of vitamin \(A_t\) for a period of 12 weeks (Curve c).](http://www.jbc.org/)
Vitamin $A_2$ in Liver—The livers of normal rats from our stock colony were found to contain 125 to 135 units of vitamin $A_1$ per gm. As may be seen from Fig. 2, where the spectra of the antimony trichloride products are given, the extract of normal rat livers shows a single absorption maximum at 620 m$\mu$. The curve for the liver extracts from vitamin $A$-deficient animals also has its absorption maximum at the same wave-length, but the peak is markedly depressed and corresponds to about 8 per cent (10.5 units per gm.) as much vitamin $A_1$ as was found in the normals.

The large group of vitamin $A$-deficient rats placed on 100 units of vitamin $A_2$ daily resumed growth promptly, and vitamin $A$ deficiency symptoms disappeared. Groups of six rats each were sacrificed after receiving vitamin $A_2$ supplementation for 3, 6, and 12 weeks. The livers from the rats after 3 weeks contained only vitamin $A_2$ to the extent of about 52 units per gm. There was no indication of a tendency to increased deposition of vitamin $A_2$ on continued feeding. Thus, the livers of rats after 6 and 12 weeks contained 36 and 40 units of vitamin $A_2$ per gm., respectively. The livers from the rats of the large group on vitamin $A_2$ supplementation for 12 weeks (Fig. 2) showed only vitamin $A_2$ (antimony trichloride absorption maximum
at 695 mp). At this time, the livers contained 76 units of vitamin A₂ per gm. The prompt rise in the vitamin A₂ level of the rat livers to values of 36 to 76 units per gm. and the maintenance at this level during continued feeding of vitamin A₂ are evidence of a systemic balance which maintains a constancy of vitamin A₂ in the liver at a given level of intake. Such a phenomenon has been previously noted by Lewis et al. (12), who report that when vitamin A-deficient rats were placed on an intake of 100 units of vitamin A₁ daily the livers establish a store of approximately 113 units per gm.

Several points emerge from these data. In vitamin A-deficient rats fed vitamin A₂, the vitamin A₂ appeared promptly in the liver and established a definite storage level. In this respect, vitamin A₂ apparently follows a pattern of systemic behavior similar to that of vitamin A₁. Only vitamin A₂ was found in the liver even after as short an interval as 3 weeks of vitamin A₂ supplementation. In the liver, vitamin A₂ appears to be a suitable biological replacement for vitamin A₁.

Vitamin A₂ in Blood—The blood serum from two large groups of normal rats was found to contain 1.0 and 0.7 units of vitamin A₁ per ml. The curve for the antimony trichloride product (Fig. 3) of the normal rat blood extract showed a maximum at 620 mp. The curve of the vitamin A-deficient rat blood extract showed a single maximum at 620 mp, but the height of the absorption curve was greatly reduced, as compared to that of the normals. The blood of these rats contained only 0.2 unit of vitamin A₁ per ml or about one-fourth to one-fifth as much vitamin A₁ as is normally present.

The blood serum from a group of six rats was examined after 6 weeks on vitamin A₂ supplementation, and it was found that vitamin A₂ had appeared in the blood (0.2 unit per ml.), although vitamin A₁ still predominated (0.3 unit per ml.). This relationship was reversed after 12 weeks of vitamin A₂ supplementation, at which time 0.2 unit per ml. of vitamin A₁ and 0.3 unit per ml. of vitamin A₂ were found in the serum. An anomalous finding is recorded in the values for the blood serum of a group of six rats chosen at random after 12 weeks on vitamin A₁ supplementation. In this serum only vitamin A₁ was present in the amount of 0.8 unit per ml. No reason for this anomaly is known.

In contrast to the rapid uptake of vitamin A₂ by the liver, the blood seemed to retain vitamin A₁ tenaciously. It would appear that 100 units of vitamin A₂ have been transported daily by the blood stream, deposited in the liver and other tissues, metabolized, and perhaps excreted for as long as

Jensen et al. (8) have found from a study of carefully prepared concentrates that the antimony trichloride product of vitamin A₁ has absorption at 695 mp equal to about 5 per cent of its absorption at 620 mp, while the antimony trichloride product of vitamin A₂ contributes absorption at 620 mp equal to about 33 per cent of its absorption at 695 mp. The relative amounts of vitamins A₁ and A₂ in mixtures were calculated on this basis.
period as 6 weeks without significantly altering the vitamin A₁ level in the blood as compared to that of vitamin A-deficient animals. The tenacity of retention of vitamin A₁ is further demonstrated by the amount of vitamin A₁ found in the blood after 12 weeks of vitamin A₂ feeding. The total amount of combined vitamin A₁ and vitamin A₂ in the blood serum after 12 weeks is nearly up to the normal level of vitamin A₁ in rat blood. However, it is apparent that prolonged periods of vitamin A₂ feeding would be necessary to replace vitamin A₁ completely, if indeed this is possible. It may be that small amounts of vitamin A₁ are stored in many tissues of the body and

![Absorption spectra of the antimony trichloride reaction products](image)

**Fig. 3.** Absorption spectra of the antimony trichloride reaction products of the extracts from blood serum of normal rats (Curve a), vitamin A-deficient rats (Curve b), depleted rats fed a supplement of 100 units daily of vitamin A₂ for 6 weeks (Curve c), and rats fed the vitamin A₂ supplement for 12 weeks (Curve d).

that upon feeding vitamin A₂ these stores are gradually replaced. The vitamin A₁ thus released perhaps finds its way into the blood stream and is responsible for its continued appearance there. However, vitamin A₂ appears in the blood stream in a reasonably constant amount and seems, over a protracted period of vitamin A₂ feeding, to be gradually replacing the vitamin A₁.

*Functional Replacement of Vitamin A₁ by Vitamin A₂*—One of the important generalities which emerges from this study is the apparent ability of vitamin A₂ to replace vitamin A₁ in general body functions. The vitamin
A-deficient rats without supplementation of vitamin A presumably would have died. When 100 units daily of vitamin A₂ were administered to each rat, all symptoms of vitamin A deficiency rapidly disappeared; growth was immediately resumed and the xerophthalmia healed. The coats became healthy looking and the rats were lively, ate well, and were in excellent condition. The livers promptly took up stores of vitamin A₂ and vitamin A₂ appeared in the blood and in the visual purple. The survival and growth on vitamin A₂ supplementation and especially the production of porphyropsin in the retinas indicate the usefulness of vitamin A₂ in those body functions in which vitamin A₁ is considered necessary.

Vitamin A₂ Reproduction—After 6 weeks on vitamin A₂ supplementation, the rats were mated; eight groups of two female rats were placed in separate cages, with one male for each group. Only half of the females became pregnant, but this percentage is not extraordinarily low in first matings. Only one of the females raised its young, although all that were pregnant delivered litters. In general, the pups were not very strong and varied markedly in weight in each litter. The pups that died had stomachs well filled with milk, showing that the failure was not in the milk supply. The one surviving litter was placed on 33 units of vitamin A₂ daily at weaning, and, during the latter part of the lactation period, the mother was put back on the usual dosage of 100 units daily. The pups had grown well at the time of sacrifice, having body weights of 50 to 80 gm. Unfortunately, the extracts of blood, livers, and retinas of the pups were cloudy and no satisfactory measurements of the antimony trichloride products of these tissues could be obtained. The loss of such a high percentage of the litters is unusual and may reflect some fault in the ability of vitamin A₂ to replace vitamin A₁. Further evidence of such a fault may be found in the wide variation in the size of the pups. The value of vitamin A₂ in reproduction and the survival of young rats is open to further experimentation.

Sex Differences in Response to Vitamin A₂—In comparison of the growth curves of the rats following vitamin A₂ supplementation, a difference was noted in the growth responses of male and female rats. For example, after 3 weeks on 100 units of vitamin A₂ the two male rats in one small group had increased about 35 gm. in body weight, whereas the four female rats had increased only 10 gm. After 6 weeks on vitamin A₂ feeding, the male rat in another small group had gained 100 gm. in body weight, whereas the five female rats had increased only 35 gm. on the average. In the large group during the 7 weeks for which weight records on vitamin A₂ supplementation are available, the twenty-eight male rats showed an average increase of 50 gm. in body weight, while the sixteen female rats showed an average increase of only 27 gm. This sex difference is also supported by evidence gained from an experiment in which two male and two female rats on a normal
intake of vitamin A₁ were fed 10,000 units daily of vitamin A₂ for 2 weeks. At the end of this time, the livers were examined separately. The livers of the male rats contained 39 and 68 units of vitamin A₁ per gm. and 171 and 254 units of vitamin A₂ per gm., respectively. The livers of the female rats contained 112 and 133 units of vitamin A₁ per gm. and 103 and 130 units of vitamin A₂ per gm., respectively. Thus, the proportion of vitamin A₂ to vitamin A₁ was much higher in the livers of the male rats than in those of the females. However, evidence for sex difference in utilization of vitamin A₂ by males and females is insufficient to be regarded as conclusive.

It should be noted here that, aside from the difference in growth response of the male and female animals, neither sex shows the weight gain that would be predicted for a daily supplementation of 100 units of vitamin A on the assumption that vitamin A₂ is equal in potency to vitamin A₁. This is in line with the observations of both Gillam et al. (7) and Jensen et al. (8), who found that the growth response to higher levels of vitamin A₂ feeding was not as great as would be predicted from the response at low levels. The latter workers also noted that very high doses of vitamin A₂ (10,000 units per day) were much more toxic than equivalent doses of vitamin A₁. This phenomenon may reflect some difference in biological response to vitamin A₂ or may be caused by some contaminant in the fresh water fish liver extract.

Dr. K. C. D. Hickman provided the original stimulus and vigorous collaboration at every step of the investigation.

The authors wish to express their thanks to Mr. E. E. Richardson of the Kodak Research Laboratories for measurements of the visual spectra, to Dr. F. P. Pingert of the Eastman Kodak Company for preparation of the sodium glycocholate solution, to Dr. A. B. McCoord of The University of Rochester School of Medicine and Dentistry for some of the vitamin A determinations, and to Mr. Ray Kesel of The University of Rochester School of Medicine and Dentistry for technical assistance.

**SUMMARY**

1. Vitamin A₂ can be incorporated into the visual purple of the albino rat, an animal normally utilizing only vitamin A₁ in this retinal pigment.
2. Upon administration of 100 "units" of vitamin A₂ daily, the liver of the albino rat promptly develops and maintains a store of vitamin A₂.
3. Upon continued feeding of vitamin A₂, the blood of the albino rat slowly increases in vitamin A₂ content while tenaciously holding to the vitamin A₁ available.
4. Vitamin A₂ appears to replace vitamin A₁ successfully in many important body functions of the rat.
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