THE REDUCTION OF RETINENE$_1$ TO VITAMIN A$_1$ IN VITRO*

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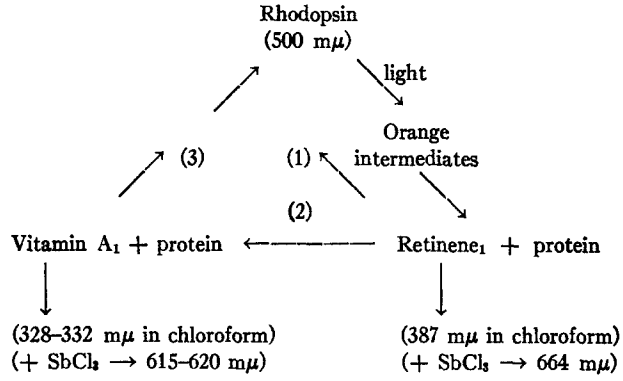
The rhodopsin system in the rods of the vertebrate retina operates as a complete cycle only in the intact eye. Here the light-sensitive rhodopsin participates with the carotenoids retinene$_1$ and vitamin A$_1$ in a closed chain of reactions of the following skeletal form:



One has only to remove the retina proper from contact with the underlying tissues which line the optic cup to abolish the synthesis of rhodopsin from vitamin A$_1$ (reaction (3) above). According to Kühne this process requires the cooperation of a living pigment epithelium (Ewald and Kühne, 1878, page 255; Kühne, 1879).

If the system is further disintegrated by bringing rhodopsin into solution in aqueous digitonin, ordinarily processes (1) and (2) are also virtually eliminated. Nothing remains but the complex succession of reactions initiated by light which transform rhodopsin into retinene$_1$ and protein.

The present investigation is concerned with reaction (2), the conversion of retinene$_1$ to vitamin A$_1$. An indication of its nature is provided in the development initiated by Morton of Liverpool. Morton and his coworkers found that on mild oxidation vitamin A$_1$ is transformed into a product which resembles retinene$_1$ in spectrum and antimony chloride reaction (Ball, Goodwin, and Morton, 1946). They have presented evidence that this product is an aldehyde;

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they believe it to be vitamin A_1 aldehyde. We have confirmed and extended these observations (Wald, 1947–48). Retinene$_1$, whether synthetic or from the retina, possesses a conjugated carbonyl group; and partition experiments, still unpublished, show that it has lost the primary hydroxyl group present in vitamin A_1. There is therefore good reason to suppose that one change in going from vitamin A_1 to retinene$_1$ is the oxidation of the alcohol radical to aldehyde.

The further correspondence between the synthetic substance and natural preparations of retinene$_1$ is not wholly complete; specifically, crude natural retinene$_1$ is a pH indicator while the synthetic substance is not. There can be no doubt however that both substances stand in the most intimate chemical relationship. It seems clear therefore that the conversion of retinene$_1$ to vitamin A_1 is or includes the reduction of the carbonyl group of retinene$_1$ to hydroxyl.

I

Cattle Retina Powder

In 1942–43 one of us succeeded in bringing the system which converts retinene$_1$ to vitamin A_1 into a cell-free preparation (Wald, 1947, 1948). Cattle retinas were frozen, desiccated in high vacuum, ground to a fine powder, and exhaustively extracted with petroleum ether, all in darkness. The residue, a dry powder which contained unaltered rhodopsin, was stirred into a brei with neutral phosphate buffer. On exposing this to light, the rhodopsin was bleached, and the retinene$_1$ formed from it was converted almost quantitatively to vitamin A_1. These experiments were interrupted by the war, and have only recently been resumed.

Such an experiment is described below; the results are shown in Figs. 1 and 2.[1]

Experiment.—Twenty cattle retinas were removed from the eyes in dim red light and were frozen at once in solid CO_2. They were desiccated at this low temperature

[1] The spectra shown in Figs. 2, 4, and 7 to 12 were drawn by the recording photoelectric spectrophotometer of Hardy, and have simply been mounted for publication. This instrument has special advantages for measuring the antimony chloride reaction with retinene and vitamin A. It is very sparing of light, an important consideration since it is now known that the blue products formed by both carotenoids with antimony chloride are highly photosensitive (*cf.* Wald, 1947–48). These products also fade even in darkness, and the speed of the Hardy instrument is therefore also advantageous. In our tests, 2.3 ml. of a saturated solution of antimony chloride in chloroform is added to 1 ml. of a chloroform solution of the test sample, with the absorption cell already in position in the spectrophotometer, and the recording is completed within about 1 minute.

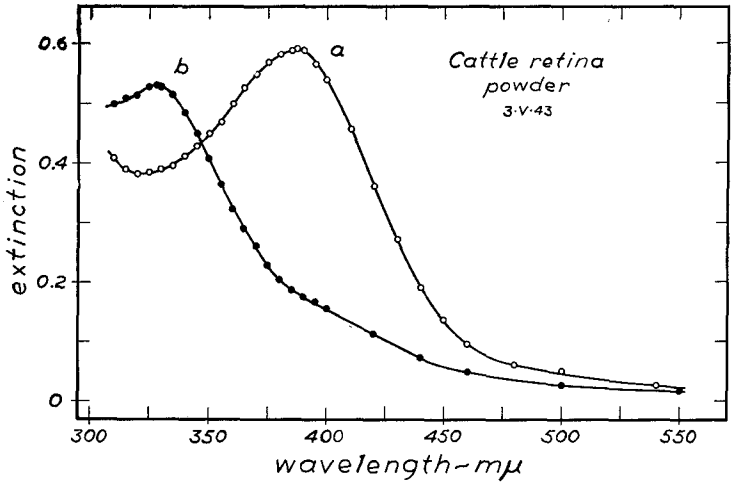


FIG. 1. The conversion of retinene$_1$ to vitamin A_1 in a cell-free brei from cattle retinas. In half this preparation the rhodopsin was destroyed in the dark with methanol at the beginning of the procedure; its extract displays the retinene$_1$ band in chloroform at 387 mμ (curve *a*). The other half was bleached with light and let stand 2 hours before being treated similarly; here the retinene$_1$ band is almost wholly replaced by that of vitamin A_1 at about 330 mμ (curve *b*). Ordinates are plotted as extinction or optical density, log I_0/I, in which I_0 is the incident and I the transmitted intensity.

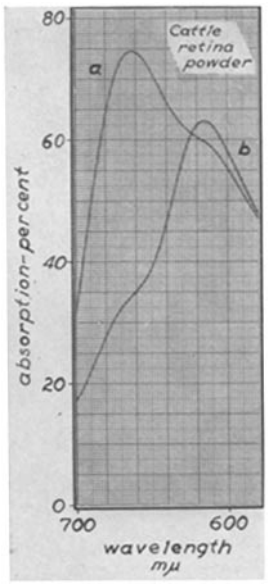


FIG. 2. The conversion of retinene$_1$ to vitamin A_1 in a cell-free brei from cattle retinas. Spectra of antimony chloride tests with the extracts of Fig. 1. The control preparation yields the absorption band at 664 mμ characteristic of retinene$_1$; the hump in the region of 610 mμ always accompanies this and does not signify vitamin A_1 (curve *a*). In the preparation bleached by light and incubated before extraction, the retinene$_1$ band is almost completely replaced by the vitamin A_1 band at 615 mμ; a trace of residual retinene$_1$ is also apparent (curve *b*).

overnight under high vacuum. The dry tissue was ground in a mortar, and divided into two equal portions. Each portion was extracted in a Soxhlet apparatus with low boiling petroleum ether (b.p. 20–40°) for 1½ hours in darkness. The solid residues were stirred in 5 ml. M/15 phosphate buffer, pH 6.95.

To one such brei 10 ml. of methanol was added in the dark. This was the control. The added methanol destroys the rhodopsin, liberating retinene$_1$, and blocks all further transformations.

Both portions were exposed to bright light, and were left in moderate light for 2 hours at room temperature. Then 10 ml. of methanol was added also to the second, experimental brei. Both were centrifuged, the solid material dehydrated by grinding with anhydrous sodium sulfate, and extracted with low boiling petroleum ether in the Soxhlet apparatus for 2 hours.

Both extracts were transferred to chloroform. Their spectra, measured with the Beckman spectrophotometer, are shown in Fig. 1. The control displays the retinene$_1$ maximum at 387 mμ; in the brei which had been bleached and incubated before methanol was added this is replaced by the vitamin A$_1$ band at about 330 mμ.

Both extracts were concentrated in chloroform and samples mixed with antimony chloride (a saturated solution in chloroform). The spectra of the resulting blue products are shown in Fig. 2. The control preparation displays the retinene$_1$-antimony chloride band at 664 mμ, the experimental the vitamin A$_1$-antimony chloride band at about 615 mμ.

The conversion of retinene$_1$ to vitamin A$_1$ in this brei was all but complete; only a trace of retinene$_1$ is evident in curves *b* of both figures.

II

Fresh Rhodopsin Solutions

Some time ago it was shown that fresh aqueous solutions of frog rhodopsin exhibit a special type of bleaching which goes further than is observed in the same solutions after a period of aging (Wald, 1937–38). This is one of the thermal or "dark" reactions which follow the exposure of rhodopsin to light. It was called dark process III in the analysis of the bleaching of rhodopsin in solution.

Bliss (1948) has now reported that the basis of this change is the formation of vitamin A$_1$. There is much else in this paper with which we do not agree, particularly the rôle assigned to what Bliss calls "acid indicator yellow" and its supposed relations with retinene$_1$. We have however confirmed the formation of vitamin A$_1$ in fresh solutions of frog rhodopsin.

An example of this reaction is described below. It can be traced directly in the original solution through the spectral changes which follow exposure to light (Fig. 3); and is further established through the antimony chloride tests with extracts of such solutions (Fig. 4).

There has been some confusion in the literature regarding the ordinary course of bleaching of rhodopsin in solution, the nature of the products formed, and

the relation of these events to what happens in the intact retina. It will aid in understanding what follows to review this situation briefly.

On irradiation of rhodopsin in neutral solution at room temperature, the first product sufficiently stable to be measured spectrophotometrically is orange

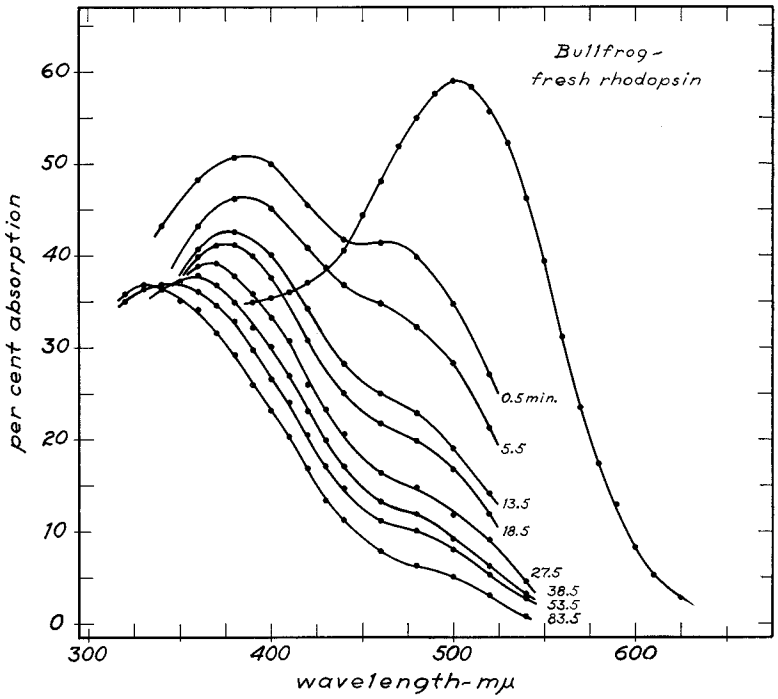


FIG. 3. Conversion of retinene$_1$ to vitamin A_1 in a fresh aqueous extract of bullfrog retinas. The original solution, protected from light during preparation and measurement, displays the rhodopsin maximum at 502 mμ. It was exposed to light for 25 seconds, and spectra thereafter measured in darkness at intervals (minutes) indicated at the right of the curves. The initial bleached spectrum shows the band of the orange intermediate at 480 mμ, superimposed on the retinene$_1$ band at 385 mμ. The 480 mμ absorption fades leaving finally a low absorption at about 500 mμ due to a trace of regenerated rhodopsin. The retinene$_1$ band gives way to a final maximum at 330 mμ due to vitamin A_1. Extract from bullfrog retinas in 1 per cent digitonin; pH 6.8; 23°C.

in color. It possesses a high absorption band in the retinene$_1$ position at about 385 mμ, and a broad hump in the visible spectrum at about 480 mμ. In darkness following the exposure to light the 480 mμ absorption falls, while the 385 mμ absorption simultaneously rises (Wald, 1937–38; dark process II).

The highly unstable material responsible for the absorption at 480 mμ is in-

cluded in what Lythgoe called "transient orange" (Lythgoe and Quilliam, 1938). We have not adopted this term because we believe it to designate not a single molecular species but a complex of intermediates between rhodopsin and retinene$_1$ + protein. Some and perhaps all of these substances change in spectrum with pH. Nor have we inserted this term heretofore in the equations of the rhodopsin cycle, because of its equivocal character, and because the status of orange intermediates in the retina as contrasted with solutions is still obscure (*cf.* Wald, 1937–38, page 828).

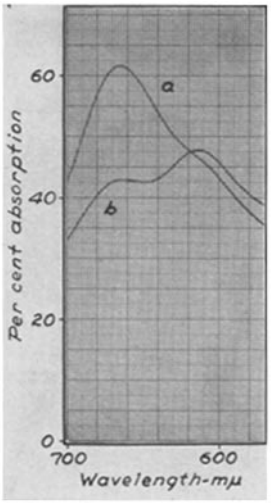


FIG. 4. Conversion of retinene$_1$ to vitamin A$_1$ in a fresh aqueous extract of bullfrog retinas. Spectra of the antimony chloride reaction with extracts of the final bleached product shown in Fig. 3 and of a control portion of the same solution destroyed in the dark with methanol. The control solution yields the retinene$_1$ band at 664 mμ (curve *a*); in the bleached and incubated solution this has largely been replaced by the vitamin A$_1$ band at about 615 mμ (curve *b*).

The final product of bleaching rhodopsin in aged solutions is a mixture of retinene$_1$ and protein, for the most part still loosely coupled together in a way that scarcely alters the retinene$_1$ spectrum. The experiments which led to this conclusion were presented some time ago (Wald, 1937–38, pages 812–813). In the equations of the rhodopsin cycle, however, we have not written retinene$_1$ as coupled to protein because in the retina, unlike aqueous solutions, retinene$_1$ is entirely liberated from protein by bleaching (Wald, 1935–36).

The retinene$_1$-protein which results from bleaching rhodopsin in solution is almost colorless when alkaline and bright yellow when acid (Chase, 1935–36).

For this reason Lythgoe called it "indicator yellow." We find its absorption maxima to lie at about 366 mμ at pH 9–9.5, 387 mμ at pH 6.7–7, and 393 mμ at pH 4–4.5. The pH lability of this complex does not depend ultimately on the coupling of retinene$_1$ to protein; for protein-free retinene$_1$, extracted with fat solvents from bleached retinas or rhodopsin solutions and brought back into aqueous solution with such a detergent as digitonin, still is a pH indicator. On the other hand the synthetic product manufactured from vitamin A_1 has lost this property; as has also natural retinene$_1$ partly purified by adsorption and elution (Wald, 1947–48).

Lythgoe described as the acid form of "indicator yellow" a material possessing a broad absorption band maximal at about 440 mμ. When rhodopsin is bleached at pH about 4 this material appears as an initial product. In light or darkness the 440 mμ maximum slowly moves toward shorter wavelengths, finally coming to rest at about 390 mμ, the maximum of acidic retinene$_1$ (Wald, 1937–38). The 440 mμ material therefore is not acidic retinene$_1$-protein but its precursor; and so is homologous with the 480 mμ precursor of retinene$_1$ in neutral solution. In Lythgoe's terminology it should be regarded as part of the "transient orange" complex, not as the acidic form of "indicator yellow."

Having once obtained retinene$_1$-protein by bleaching rhodopsin in solution, one can by treatment with strong acids convert it to highly colored products with absorption maxima at 440 mμ or longer wavelengths. The same can be done with retinene$_1$ extracted from such solutions or from retinas with fat solvents and therefore protein-free; even here retinene$_1$ may still be coupled with other molecules. Ball *et al.* (1948) have reported obtaining such products from synthetic retinene$_1$ on treatment with acids in the presence of certain proteins, amino acids, and aromatic amines. These are the artefacts which Bliss (1948) calls "acid indicator yellow." They have an interest of their own, but are neither precursors of vitamin A_1 nor do they play any other direct rôle in the visual processes.

The bleaching of a fresh solution of neutral rhodopsin is shown in Fig. 3. The original spectrum, measured before exposure to light, possesses the rhodopsin maximum at 500 mμ. Immediately following irradiation the spectrum displays the broad maximum at about 480 mμ of the orange intermediate, superimposed on a high retinene$_1$ band at about 385 mμ. In darkness, the 480 mμ maximum slowly declines, leaving finally a very low absorption at about 500 mμ due to a little regenerated rhodopsin. Instead of the 385 mμ maximum simultaneously growing, as it would have done in aged solutions, it moves toward shorter and shorter wavelengths, reaching a final position at about 330 mμ. This is the band of vitamin A_1. In the final spectrum a raised absorption in the region of 380 mμ marks a residue of unchanged retinene$_1$.

Antimony chloride tests with extracts of such solutions confirm the trans-

formation of retinene$_1$ to vitamin A_1. Half of the original preparation which yielded Fig. 3 had been kept dark as a control, and was destroyed in the dark with methanol. Its extract, mixed with antimony chloride, yielded curve *a* of Fig. 4, displaying the 664 mμ maximum of retinene$_1$ alone. The final product of the experiment shown in Fig. 3 was treated similarly. It yielded curve *b* of Fig. 4, showing beside a residue of retinene$_1$ the dominant absorption at 615 mμ due to newly formed vitamin A_1.

Experiment[2].—Retinas of two bullfrogs were extracted by stirring in 2 per cent aqueous digitonin solution for 30 minutes. The mixture was centrifuged 15 minutes at 11,500 R.P.M. and the clear extract poured off. This was diluted with an equal volume of M/15 phosphate buffer, pH 6.84, and divided into two equal portions. All these operations were carried out in dim red light.

Half the solution was set aside in the dark as a control; the remainder was used in the experiment shown in Fig. 3. The latter was irradiated 3 hours after the beginning of dissection and 1½ hours after the beginning of extraction. Spectra were measured with the Beckman spectrophotometer, a slower instrument than is desirable for work of this type. Nevertheless the spectra show clearly the principal events which follow exposure of the rhodopsin to light.

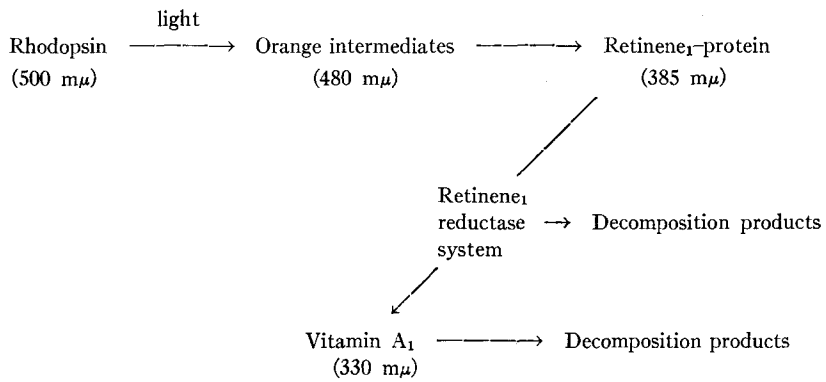
For the antimony chloride tests shown in Fig. 4, methanol was added to a concentration of 60 per cent to both the bleached solution and the dark control. Both solutions were extracted by shaking with petroleum ether. The extracts were transferred to chloroform and mixed with antimony chloride solution in the spectrophotometer. The spectra of the blue products were recorded within the first minute after mixing the reagents.

Fresh solutions of rhodopsin exhibit a further change, not shown in Figs. 3 and 4. This is the slow destruction of vitamin A_1. Within 10 hours after the exposure to light, all carotenoids have usually disappeared from the solution. The direct spectrum shows no evidence of either retinene$_1$ or vitamin A_1, and the antimony chloride test is negative.

Finally, in such fresh solutions the enzyme system itself disintegrates. Within a period of several hours at room temperature the preparation loses most of its capacity to convert retinene$_1$ to vitamin A_1.

The changes known to occur in fresh rhodopsin solutions can therefore be formulated as follows:—

[2] In this and all subsequent experiments the frogs were dark-adapted overnight before removal of the retinas. The dissection and all other preparatory operations were performed either in dim red light, to which rhodopsin is insensitive, or in darkness. In each experiment these conditions were maintained to the point at which it is explicitly stated that solutions or tissues were exposed to light In all cases this was the white light from a tungsten filament lamp.



The significance of the term "retinene$_1$ reductase system" will become evident below. This system alone presents a number of opportunities for side reactions.

In so complicated a mixture of interdependent processes, it is almost impossible to interpret reliably the effects of change of temperature, pH, or other conditions. Before one can examine with confidence the properties of the transformation of retinene$_1$ to vitamin A_1, it will be necessary to isolate this reaction to a reasonable degree and to stabilize its enzyme system.

III

Rod Outer Limbs

We have described the conversion of retinene$_1$ to vitamin A_1 in a cell-free brei and in aqueous detergent solutions. In order to analyze such systems further one would ordinarily fractionate them in the attempt to isolate their essential components. We had already begun such experiments, when the investigation took a new turn with the discovery that the enzyme system is already fractionated anatomically in the structure of the retinal rods.

The vertebrate rod is composed of two sections, the so called inner and outer limbs or segments. The inner limb is not very different from a nerve cell, though it makes synaptic connection only at one end since it is the first member in an excitation chain. This portion of the rod contains the nucleus and is presumably the seat of the main vegetative functions.

The outer limb is a complex cellular outgrowth, which contains all the rhodopsin to be found in the retina. Within a sheath of neurokeratin, it appears to be composed of alternate layers of protein and lipid, both highly oriented in what is essentially a crystalloidal structure (Schmidt, 1938). In the frog, in which the rods are unusually large, the outer segment is a cylinder about 6 to 9 micra wide and about 50 micra long. Within this small compass is enclosed the whole of the photoreceptor process. This must represent about as high a degree of isolation of a physiological function as the organism offers.

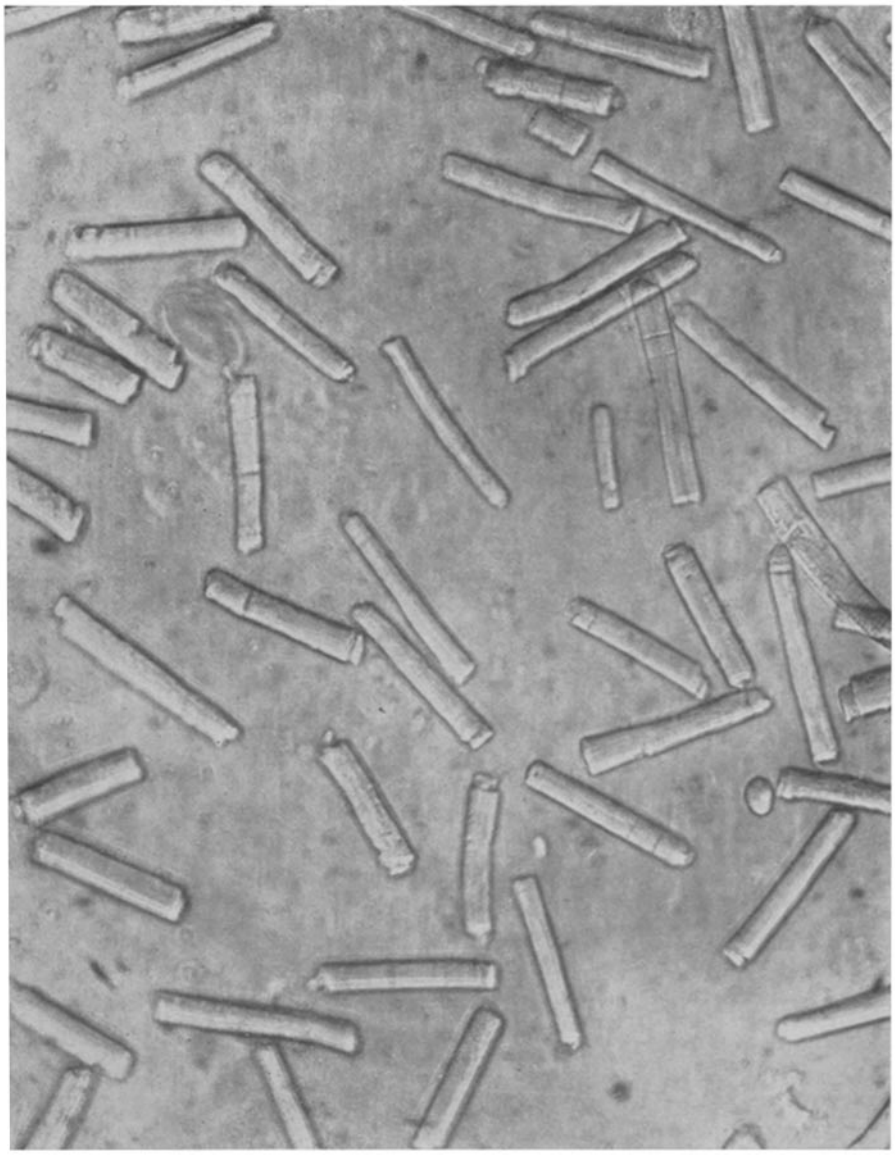


FIG. 5. Microphotograph of rod outer segments suspended in Ringer solution. Magnification about 500. The longitudinal striations which can be seen in most of the outer limbs are characteristic of fresh preparations, and probably are evidence of a fibrillar structure in the outer limb. Later, cross-striations appear and eventually dominate the structure; the first of these also are visible in the photograph.

If one removes the retina from a frog eye into Ringer solution with all possible care, the solution examined under a microscope is found to contain large numbers of isolated rod outer limbs. These have been broken off in the course of dissection, just at the juncture with the inner limbs. Thereafter they maintain their integrity for considerable periods (Fig. 5).

By special methods one can remove larger numbers of outer segments from the retina. Dense suspensions of them have been used by Lythgoe (1937) and by Saito (1938) to prepare rhodopsin solutions.

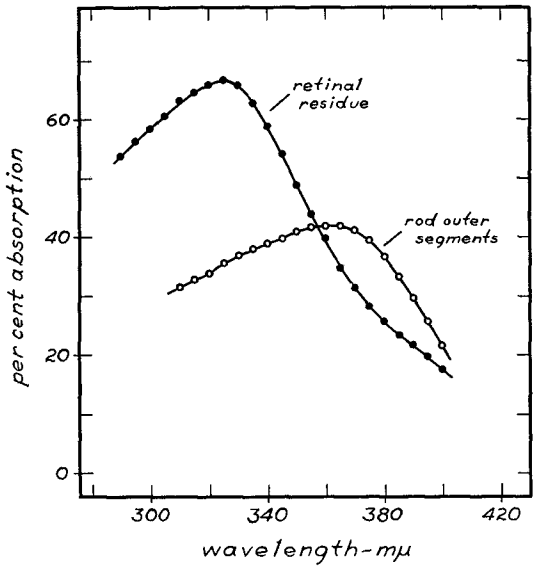


FIG. 6. Rod outer segments detached from the retina do not convert their retinene$_1$ to vitamin A_1. Numbers of rod outer segments had been scraped from frog retinas and left suspended with the retinal remainders during a period of bleaching and incubation. They were then isolated, and they and the retinal residues extracted separately with hexane. The spectra of these extracts are shown. The detached rods yield the band at about 365 mμ of retinene$_1$; the retinal residues to which outer limbs had remained attached yield the band at 325 mμ of vitamin A_1.

Such a suspension of rod outer limbs can be separated almost completely from other retinal tissues. When prepared under deep red light it contains a large quantity of rhodopsin. On exposure to white light this bleaches to a yellow-orange color. Unlike whole retinas, however, such isolated outer segments do not go on fading to colorlessness, the change associated with the conversion of retinene$_1$ to vitamin A_1. On extracting the bleached outer limbs even after hours of incubation one finds only retinene$_1$. The transformation of retinene$_1$ to vitamin A_1 does not occur in the isolated outer limb.

This situation is not changed if the outer limb is left suspended in the same medium with the remainder of the retina. It is enough to break the outer segment away from the underlying tissue to abolish its capacity to form vitamin A$_1$. On the other hand those outer limbs which remain attached to the retina continue to convert their retinene$_1$ to vitamin A$_1$ as before.

These relations are demonstrated in the following experiment, the results of which are shown in Fig. 6.

Rod Suspension.—The retinas of dark-adapted frogs (*Rana pipiens*) are prepared in Ringer solution in dim red light. They are scraped by gently stroking the posterior surface which bears the receptor cells with a spatula or fine forceps. The retinal remainders are lifted into a separate test tube after scraping; usually they still retain about half their original content of outer limbs. The suspension of isolated outer segments is filtered through three layers of cheesecloth to remove shreds of other tissues. The suspension is centrifuged, and the rods used as desired.

Experiment.—Outer limbs were scraped from the retinas of nine dark-adapted frogs. In this instance they were left suspended with the other retinal tissues in Ringer solution. The whole suspension was exposed to white light, then allowed to stand for 1 hour at room temperature. The detached outer limbs were now filtered from the retinal remainders. Both tissues were dehydrated by grinding with anhydrous sodium sulfate, and were extracted with hexane. The spectra of these extracts are shown in Fig. 6.

The detached outer limbs yielded the retinene$_1$ band at about 365 mμ in hexane, with just a suggestion of the vitamin A$_1$ maximum at 325 mμ. The retinal remainders yielded the vitamin A$_1$ band alone. That is, though both tissues were incubated in the same medium, only the rods which had remained attached to the underlying retinal tissues had formed vitamin A$_1$. Judging by the ratios of the absorptions of retinene$_1$ and vitamin A$_1$ in this experiment, about twice as many rods had remained attached to the retina as had been broken away.

IV

The Coenzyme of Retinene$_1$ Reduction

If whole retinas are thoroughly mashed with a glass rod in Ringer solution or phosphate buffer, almost all the outer limbs are detached from other tissues in the process. Yet the suspension which results efficiently converts retinene$_1$ to vitamin A$_1$. The breaking up of the retinal tissue by grinding releases substances which promote this process in the outer limbs.

Presumably these factors are carried to the outer limbs in solution in the suspension fluid. If such a suspension is centrifuged and the supernatant liquid poured off, the solid residue which contains all the rhodopsin has lost almost completely the power to form vitamin A$_1$. On re-adding the supernatant it regains this capacity (Fig. 8).

A clear, colorless water extract of retinas added to isolated rod outer limbs constitutes a complete system for converting retinene$_1$ to vitamin A$_1$. Indeed

it is not necessary for this that the rods maintain their normal structure. They may be frozen, desiccated, ground, and exhaustively extracted with petroleum ether; and the residue from this treatment, suspended in a water extract of

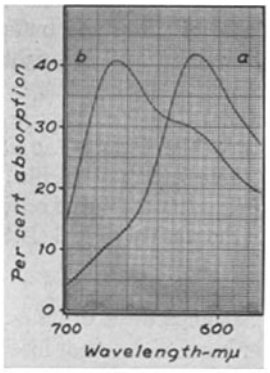


FIG. 7

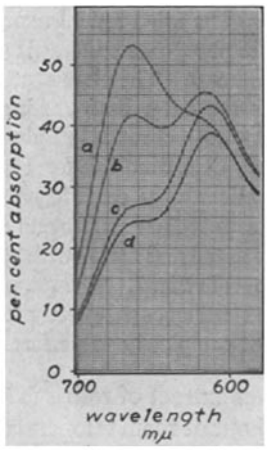


FIG. 8

FIG. 7. Rod outer segments suspended in a water extract of retina convert retinene$_1$ to vitamin A_1; the washed retinal residue does not. Outer limbs were isolated and were frozen, dried, and preextracted with petroleum ether. The portions of retina from which they were taken were extracted with neutral phosphate buffer, and the rod residues suspended in the extract. They and the washed retinal tissue were irradiated and incubated. The spectra of the antimony chloride reactions with their extracts are shown. That from the washed retinal tissue displays the band of retinene$_1$ (curve *b*); while the outer segment preparation suspended in retinal washings has converted its retinene$_1$ entirely to vitamin A_1 (curve *a*). This result is just the reverse of that shown in Fig. 6; the difference is that here a water extract has been transferred from the retinal residues to the detached outer limbs.

FIG. 8. Washed retina is reactivated by returning the water extract; the water-soluble factor is relatively heat-stable. Washed retinal tissue had been suspended in a number of solutions, bleached, and incubated for 1 hour. The spectra of the antimony chloride tests with extracts of the final products are shown. The washed tissue suspended in phosphate buffer had not converted its retinene$_1$ to vitamin A_1 (*a*); suspended in a water extract of retina which had been held in boiling water for 7 minutes it performed about a half conversion (*b*); in retinal extract kept for 7 minutes on ice (*c*) or at room temperature (*d*) the conversion was nearly complete.

retina, still transforms its retinene$_1$ into vitamin A_1. On the other hand the retinal tissue from which the water extract was taken no longer can perform this conversion.

The following experiment, the results of which are shown in Fig. 7, demonstrates these relationships.

Experiment.—Retinas of ten dark-adapted frogs, dissected out under red light, were scraped as described above, and the rod outer limbs filtered from the retinal remainders. Both tissues were desiccated under vacuum at low temperature. The dried rods were extracted by shaking with three portions of petroleum ether, each time for 20 minutes. The dried retinal remainders were ground thoroughly with a glass rod in 1 ml. M/15 phosphate buffer, pH 6.8, for 15 minutes; then centrifuged for 15 minutes at 4000 R.P.M. The clear supernatant was poured off and used as a suspension medium for the outer limb residues. An equal volume of phosphate buffer was added to the washed retinal residues. Both mixtures were exposed to bright light for 1 minute, then were left for 1¼ hours in moderate light at room temperature. Both suspensions were centrifuged and the solid material dehydrated by grinding with anhydrous sodium sulfate and extracted with 3.5 ml. chloroform. The extracts were concentrated and tested with antimony chloride. The spectra of the blue products are shown in Fig. 7. The rod powder suspended in a water extract of retina had converted its retinene$_1$ completely to vitamin A$_1$; while the washed retinal tissue had failed almost completely to perform this conversion.

The water extract of retina is highly unstable. It loses most of its activity within an hour at room temperature. On the other hand it does not lose all its activity on being brought to 100°C. for as long as 7 minutes, though this amount of heating should be enough to destroy most enzymes.

These relations are illustrated in the following experiment, the results of which are shown in Fig. 8.

Experiment.—Retinas of twelve frogs were dissected in red light and ground thoroughly for 15 minutes in 2 ml. of M/15 phosphate buffer, pH 6.84. The suspension was divided into four equal portions and all were centrifuged at 4000 R.P.M. The supernatants were poured off, mixed, and redivided into three equal portions. We had therefore prepared four portions of washed retina and three portions of retinal extract.

The latter were kept for 7 minutes, one on ice, one at 23°C., and the third in boiling water. All were brought to the same temperature. Then these extracts were added to three of the samples of washed retina, while to the fourth an equal volume of phosphate buffer was added. All were stirred and exposed together to white light, and were left at room temperature for 1 hour. All were centrifuged, the liquid poured off, and the solid residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride.

The spectra of the resulting blue products are shown in Fig. 8. The water extracts of retina which had been kept on ice (curve *c*) or at room temperature (curve *d*) displayed strong activity; in the washed retina to which they were added almost all the retinene$_1$ had been converted to vitamin A$_1$. The boiled extract also had been fairly active; here the retinene$_1$ was about half converted (curve *b*). The washed retinal tissue to which simple buffer had been added had failed to form vitamin A$_1$ (curve *a*).

The ease and completeness with which the water-soluble factors are washed from the retina and their relative stability toward heat argued against the

likelihood that they are enzymes. Our experiments suggested rather that we were dealing with relatively small and simple molecules, perhaps of the nature of substrates and coenzymes. In this case, however, there is no reason to expect these substances to be confined to the retina alone. One could look forward to finding them in other tissues.

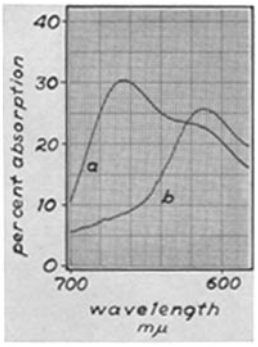


FIG. 9

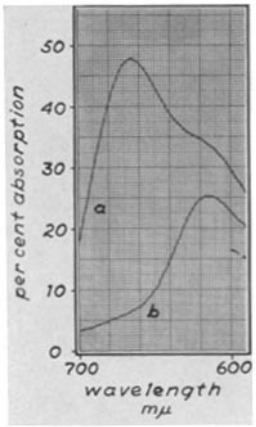


FIG. 10

FIG. 9. Boiled muscle juice reactivates washed retina. Equal portions of water-extracted retina were suspended in phosphate buffer and in a boiled juice of frog muscle. The suspensions were exposed to light, left at room temperature for 1 hour, and extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. The washed tissue in buffer solution did not convert its retinene$_1$ to vitamin A_1 (curve *a*); that suspended in boiled muscle juice did so completely (curve *b*).

FIG. 10. Boiled muscle juice activates isolated rod outer limbs. Equal portions of detached rod outer segments were suspended in phosphate buffer and in a boiled juice of frog muscle. The suspensions were exposed to light, left at room temperature for 1 hour, and the residues extracted with petroleum ether. The spectra of the antimony chloride tests with these extracts are shown. Outer limbs in buffer had failed to convert their retinene$_1$ to vitamin A_1 (curve *a*); those suspended in boiled muscle juice had done so completely (curve *b*). The relatively low content of vitamin A_1 shown in curve *b* is due to its destruction in preparations of this type.

To explore this possibility we prepared a boiled extract of frog muscle and added it to a preparation of washed retina. It promoted the conversion of retinene$_1$ to vitamin A_1 as efficiently as did the best of our retinal extracts. The boiled muscle juice was equally effective in promoting the formation of vitamin A_1 by isolated outer limbs. This left no doubt that the factors we sought are present in muscle as in retina, and are thermostable.

The action of boiled muscle juice on washed retina and on outer limbs is demonstrated in the following experiment and in Figs. 9 and 10.[3]

Experiment.—Retinas of twelve frogs prepared in red light were scraped thoroughly, the detached outer limbs filtered from the retinal remainders, and the latter ground in about 1.5 ml. of M/15 phosphate buffer, pH 6.84. The outer limb and retinal suspensions were each divided into two equal portions and centrifuged 15 minutes at 4000 R.P.M. The supernatants were discarded. This procedure netted us two portions of isolated outer limbs and two of washed retina.

A boiled muscle juice was prepared by mincing the gastrocnemius and sartorius muscles of one frog, and grinding them in a mortar with 10 ml. of a 1:1 mixture of Ringer solution and pH 6.84 phosphate buffer. This brei was filtered through cheesecloth, and the filtrate brought to a boil. The clear liquid was decanted and rapidly chilled to room temperature.

2 ml. of boiled muscle juice was added to one portion of outer limbs and to one of washed retina. To the remaining portions of both types of tissue was added 2 ml. of buffer-Ringer mixture. All four samples were stirred into suspension, exposed to bright white light, and left at room temperature for 1 hour. All were centrifuged, the liquid poured off, and the solid material dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Figs. 9 and 10.

Washed retina suspended in boiled muscle juice converted its retinene$_1$ quantitatively to vitamin A$_1$ (Fig. 9, curve *b*); the same tissue in buffer solution failed to perform this conversion (curve *a*). Similarly, isolated outer limbs in boiled muscle juice transformed their retinene$_1$ completely to vitamin A$_1$ (Fig. 10, curve *b*), while they did not go through this reaction at all in buffer solution (curve *a*).

We have noted above that in the conversion of retinene$_1$ to vitamin A$_1$ an aldehyde group is reduced to hydroxyl. In this reaction therefore retinene$_1$ acts in the rôle of hydrogen acceptor. Boiled muscle juice contains a number of substances which might donate hydrogen for this process; it is famous also for its content of a major coenzyme of hydrogen transfer, cozymase, coenzyme I, or DPN.

A preparation of washed retina to which DPN was added still failed to convert retinene$_1$ to vitamin A$_1$. One would expect such a preparation to have retained at least a fraction of all its original enzymes; it may however have lost essential substrates.

[3] Fig. 10 illustrates another relation of some interest. Retinene$_1$ is reasonably stable in suspensions of rod outer limbs; but when these are placed in circumstances in which they can form vitamin A$_1$, the vitamin is destroyed within several hours after its formation. This accounts for the low level of vitamin A$_1$ compared with retinene$_1$ shown in the figure. It will be recalled that a similar destruction of vitamin A$_1$ occurs in fresh rhodopsin solutions.

We therefore added DPN to washed retina combined with various concentrations of the potential hydrogen donors, lactic and succinic acids. In some instances small amounts of vitamin A_1 seem to have formed, but none of these preparations produced it efficiently.

Failing a suitable substrate temporarily, we tried the effect of already reduced cozymase (DPN-H_2). A sample of DPN was reduced with sodium hydrosulfite ($Na_2S_2O_4$), and the excess hydrosulfite oxidized away by blowing air through the solution (Green and Dewan, 1937). The reduced cozymase was added to a preparation of washed retina. It converted the retinene$_1$ formed on bleaching almost wholly to vitamin A_1. As might be expected of such a process, it was if anything aided by the total exclusion of oxygen.

The action of reduced DPN on washed retina is demonstrated in the following experiment and in Fig. 11.

Experiment.—Retinas of five frogs were prepared in red light and ground in M/15 phosphate buffer, pH 6.84. The suspension was divided into two equal parts and both were centrifuged 15 minutes at 4000 R.P.M. The fluid was discarded and the residues resuspended in 1 ml. of pH 6.84 buffer. These suspensions of washed retina were transferred to the bulbs of two Thunberg tubes.

Reduced DPN was prepared according to the method of Green and Dewan (1937). A commercial preparation of DPN (Schwarz, 60 per cent active) was made up in 5 ml. to a concentration of 0.15 per cent DPN. To this was added 2.5 ml. of a 1:1 mixture of 0.5 per cent $NaHCO_3$ and 0.2 per cent $Na_2S_2O_4$. The excess hydrosulfite was removed by bubbling air through the solution. A control solution was prepared by repeating the entire procedure with the same reagents but without DPN.

2.5 ml. of the reduced DPN and of the control mixture were placed in the bodies of the Thunberg tubes. The tubes were alternately evacuated and washed through with nitrogen three times and finally were closed under vacuum. The contents were then mixed and exposed to bright white light. They were left at room temperature in moderate light for 1¼ hours. The contents were then centrifuged, the residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Fig. 11.

Washed retina suspended in the control salt mixture had failed to convert its retinene$_1$ to vitamin A_1 (curve *a*); but that to which reduced DPN was added had performed this conversion almost completely (curve *b*).

There remained the problem of finding a substrate which in the presence of washed retina or isolated rod outer limbs would reduce DPN. As noted above, neither succinic nor lactic acid fulfills this function. We have however found a first such substrate in fructosediphosphate.

A commercial preparation of hexosediphosphate promotes some conversion of retinene$_1$ to vitamin A_1 by washed retina or outer limbs even without the addition of DPN. Presumably this involves some residue of DPN retained by

the retinal tissue. But with added DPN the action goes much further, and yields a very efficient formation of the vitamin.

These relations are demonstrated in the following experiment, the results of which are shown in Fig. 12.

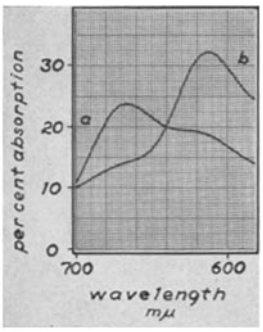


FIG. 11

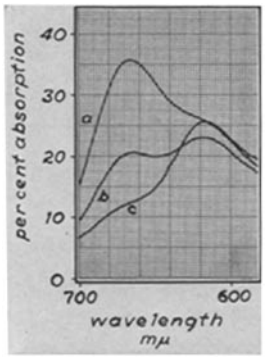


FIG. 12

FIG. 11. The action of reduced cozymase on washed retina. Equal portions of a preparation of water-extracted frog retina were suspended in a solution containing reduced DPN and in an otherwise identical solution lacking only the DPN. Both suspensions were bleached in the light, incubated, and the residues extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. The control preparation yielded retinene$_1$ alone (curve *a*); while in the washed retina to which reduced DPN had been added this had been converted almost completely to vitamin A$_1$ (curve *b*).

FIG. 12. The action of DPN and fructosediphosphate on isolated rod outer limbs. Equal portions of a preparation of rod outer segments were suspended in (*a*) phosphate buffer, pH 6.84; (*b*) a preparation containing fructosediphosphate in phosphate buffer; and (*c*) the latter mixture to which DPN was also added. The suspensions were made anaerobic, exposed to light, incubated for 2 hours, and the rod residues extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. Rod outer limbs in buffer mixture yielded only retinene$_1$ (curve *a*); with hexosediphosphate added they had converted about half their retinene$_1$ to vitamin A$_1$ (curve *b*); with both hexosediphosphate and DPN added, the conversion to vitamin A$_1$ was nearly complete (curve *c*).

Experiment.—A suspension of rod outer limbs was isolated from the retinas of eleven frogs. It was divided into three equal portions. These were centrifuged, the liquid poured off, and the rods resuspended in 1 ml. portions of M/15 phosphate buffer, pH 6.84. The suspensions were transferred to the bulbs of three Thunberg tubes.

To the body of one Thunberg tube was added 2.25 ml. of the phosphate buffer as

control; to the second, 2 ml. of 0.017 molar fructosediphosphate:[4] and 0.25 ml. of the phosphate buffer; and to the third tube this same mixture to which had been added 5 mg. of DPN powder (about 5×10^{-7} mols).

All the tubes were evacuated and flushed through with nitrogen several times, then shut off under vacuum. The contents were mixed, exposed to white light, and left in moderate light at room temperature for 2 hours. The contents were then centrifuged and the solid residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Fig. 12.

Isolated rod outer limbs suspended in buffer mixture had as before failed to convert their retinene$_1$ to vitamin A_1 (curve *a*). In the presence of a crude preparation of fructosediphosphate they had performed a partial conversion (curve *b*). With fructosediphosphate and added DPN the conversion to vitamin A_1 was very nearly complete (curve *c*).

V

DISCUSSION AND CONCLUSIONS

We have shown that the conversion of retinene$_1$ to vitamin A_1 is a coupled reduction, for which cozymase (DPN) acts as coenzyme and fructosediphosphate can act as substrate. The essential process is the transfer of two atoms of hydrogen by DPN from a hydrogen donor to retinene$_1$, reducing its aldehyde group to the primary alcohol group of vitamin A_1:

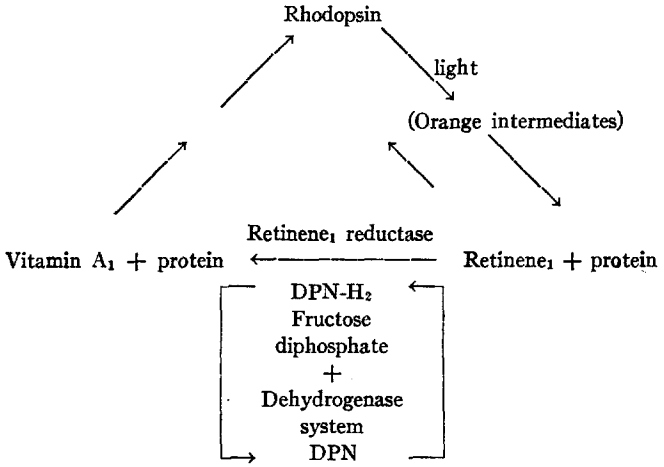
$$\text{Retinene}_1 + \text{DPN—H}_2 \xrightarrow[\text{reductase}]{\text{Retinene}_1} \text{Vitamin A}_1 + \text{DPN}$$

We have not yet demonstrated that the reduction of retinene$_1$ with DPN-H_2 requires a special enzyme, but this is made so probable by all that is known of such reactions that one is justified in assuming it to be the case. This apoenzyme, which we will call retinene$_1$ reductase, is present in the outer segment of the rod.

The system which reduces retinene$_1$ must work in conjunction with a second system which reduces DPN. This also requires a special apoenzyme which is

[4] The fructosediphosphate used in these experiments was a preparation of the dibarium salt supplied by the Schwarz Laboratories of New York City. It contained 8.4 per cent P, and on this basis was about 83 per cent pure. To prepare it for use, it was taken up in HCl, the barium precipitated with sodium sulfate, and the solution neutralized with sodium hydroxide.

present in the rod outer limb. The organization of the total process can therefore be written:



This development lifts the rhodopsin cycle out of its former isolation, coupling it with one of the most general processes in cellular metabolism, the transfer of substrate hydrogen by cozymase. It introduces also a new vitamin relation. The rhodopsin system, long known to contain vitamin A$_1$ as a direct participant, is now seen to depend secondarily upon a member of the vitamin B complex, the antipellagra factor nicotinic acid amide, contained in DPN.

It is not necessary that fructosediphosphate, simply because it is effective, be regarded as the hydrogen donor in the reduction of retinene$_1$. The preparations of outer limbs or washed retina which catalyze this reaction probably are able also to degrade fructosediphosphate fermentatively, and one of its derivatives probably serves as the immediate source of hydrogen in our reaction. Among such possibilities one thinks first of 3-phosphoglyceraldehyde, the substance which reduces DPN in the alcohol and lactic acid fermentations, and the immediate product of the action of aldolase on fructosediphosphate.

Since the transformation of retinene$_1$ to vitamin A$_1$ is a reduction, place must be found elsewhere in the closed rhodopsin cycle for an oxidation. We have stressed heretofore the conjugation of carotenoid with protein in the synthesis of rhodopsin, and its cleavage from protein following the exposure of rhodopsin to light. It is now apparent that one or the other of these processes must include also an oxidative reaction.

This is not however the direct reoxidation of vitamin A$_1$ to retinene$_1$. A curious and significant property of the retinene$_1$ reductase system is that it operates irreversibly. In the intact isolated retina and in the *in vitro* systems we have described above, vitamin A$_1$ accumulates as the sole carotenoid end-prod-

uct. We have found no indication whatever of the oxidation of vitamin A_1 to retinene$_1$, except through the roundabout intermediation of rhodopsin in the complete retinal cycle.

It may be an advantage to the organism that the retinene$_1$ reductase system behaves in this fashion. Were vitamin A_1 oxidized directly to retinene$_1$, this would be a means of withdrawing the vitamin from circulation and accumulating large stores of retinene$_1$ for which there is probably no function outside the retina. It is an attractive hypothesis that in the retina the utilization of vitamin A_1 begins with its conjugation to the protein moiety of rhodopsin, and that it is oxidized only after this has occurred. This would then be a self-limiting process, restricted to the amount of rhodopsin protein present in the rod outer segment.

Since the rod outer segment contains retinene$_1$ and the apoenzymes for reducing both it and DPN, we may assume that in its normal position in the retina it contains also DPN and a suitable hydrogen donor. That is, it probably comes to lack DPN and substrates only after being detached from the rest of the retina. The isolated outer limb is no more than a fragment of a cell, broken off at one end. Such relatively small, water-soluble molecules as DPN and sugar derivatives probably leak from the detached outer segment into the suspension medium, until their concentrations have fallen too low to be effective. In this sense the isolated outer limbs can themselves be regarded as a "washed" tissue. In the intact retina they probably contain all that is needed to reduce retinene$_1$. There is no present reason to believe that this process normally demands a migration of substances between the outer segments and the underlying retinal tissues.

It is now clear that the fresh rhodopsin solutions which are able without supplementation to convert their retinene$_1$ to vitamin A_1 must possess not only retinene$_1$ reductase and DPN, but the enzyme system and substrates for reducing DPN. The deterioration of such solutions could have multiple causes. Probably the primary cause of their decay, however, is the loss of DPN. It is now well known that this nucleotide is rapidly destroyed in autolysates from a wide variety of organs. Preparations of brain tissue, to which retina is closely related, are particularly active in this regard (Handler and Klein, 1942). We are now exploring the possibility that the reduction of retinene$_1$ can be stabilized in fresh solutions of rhodopsin and restored in aged solutions by the addition of DPN.

SUMMARY

In the surviving vertebrate retina the retinene$_1$ liberated by bleaching rhodopsin is converted quantitatively to vitamin A_1. Recent chemical studies have indicated that in this process the aldehyde group of retinene$_1$ is reduced to the primary alcohol group of vitamin A_1 (Morton; Wald).

Some time ago we brought this reaction into a cell-free brei prepared from cattle retinas. The retinas were frozen, desiccated, ground, and exhaustively extracted with petroleum ether; the resulting powder, stirred in neutral buffer solution and exposed to light, converted its retinene$_1$ completely to vitamin A$_1$.

Some time ago also we observed that fresh rhodopsin solutions exhibit a special type of fading in darkness following exposure to light, which is absent from the same solutions after aging. We have confirmed Bliss's identification of this reaction as the conversion of retinene$_1$ to vitamin A$_1$.

The system which reduces retinene$_1$ is fractionated anatomically in the retinal rods. The outer segments of the rods, broken off from the underlying retinal tissue, are unable to convert their retinene$_1$ to vitamin A$_1$. In the presence of a water extract of crushed retina they do perform this conversion. On the other hand the retinal tissue from which a water extract was taken has lost this capacity. Such washed retinal tissue is reactivated by returning the washings to the solid material.

The activating effect of retinal washings on isolated outer limbs or washed retina is duplicated by a boiled muscle juice. This in turn can be replaced by reduced cozymase (reduced coenzyme I; DPN-H$_2$); or by a mixture of DPN and fructosediphosphate.

The conversion of retinene$_1$ to vitamin A$_1$ is therefore a reduction in which two atoms of hydrogen are transferred to retinene$_1$ from reduced cozymase. It is assumed that this reaction is catalyzed by an apoenzyme, retinene$_1$ reductase, present in the rod outer limb. This process is coupled with a second system in the outer segment which reduces DPN, using hexosediphosphate or one of its derivatives as hydrogen donor. This action of DPN brings a member of the vitamin B complex, nicotinic acid amide, into an auxiliary position in the rhodopsin system.

In the isolated retina or *in vitro* systems the reduction of retinene$_1$ proceeds irreversibly. Yet this reduction must be balanced by an oxidative process elsewhere in the rhodopsin cycle, since through rhodopsin as intermediate vitamin A$_1$ regenerates retinene$_1$.

REFERENCES

Ball, S., Collins, F. D., Morton, R. A., and Stubbs, A. L., Chemistry of visual processes, *Nature*, 1948, **161**, 424.

Ball, S., Goodwin, T. W., and Morton, R. A., Retinene-vitamin A aldehyde, *Biochem. J.*, 1946, **40**, p. lix.

Bliss, A. F., The mechanism of retinal vitamin A formation, *J. Biol. Chem.*, 1948, **172**, 165.

Chase, A. M., Anomalies in the absorption spectrum and bleaching kinetics of visual purple, *J. Gen. Physiol.*, 1935–36, **19**, 577.

Ewald, A. and Kühne, W., Untersuchungen über den Sehpurpur, II. Entstehung der Retinafarbe, *Untersuch. physiol. Inst. Univ. Heidelberg*, 1878, **1**, 248.

Green, D. E., and Dewan, J. G., The reversible oxidation and reduction of coenzyme I, *Biochem. J.*, 1937, **31,** 1069.

Handler, P., and Klein, J. R., The inactivation of pyridine nucleotides by animal tissues *in vitro*, *J. Biol. Chem.*, 1942, **143,** 49.

Kühne, W., Chemische Vorgänge in der Netzhaut, in Handbuch der Physiologie, (L. Hermann, editor), Leipzig, F. C. W. Vogel, 1879, **3,** pt. 1, 312.

Lythgoe, R. J., The absorption spectra of visual purple and of indicator yellow, *J. Physiol.*, 1937, **89,** 331.

Lythgoe, R. J., and Quilliam, J. P., The relation of transient orange to visual purple and indicator yellow, *J. Physiol.*, 1938, **94,** 399.

Saito, Z., Isolierung der Stäbchenaussenglieder und spektrale Untersuchung des daraus hergestellten Sehpurpurextraktes, *Tohoku J. Exp. Med.*, 1938, **32,** 432.

Schmidt, W. J., Polarisationsoptische Analyse eines Eiweiss-Lipoid-Systems, erläutert am Aussenglied der Sehzellen, *Kolloid-Z.*, 1938, **85,** 137.

Wald, G., Carotenoids and the visual cycle, *J. Gen. Physiol.*, 1935–36, **19,** 351.

Wald, G., On rhodopsin in solution, *J. Gen. Physiol.*, 1937–38, **21,** 795.

Wald, G., The photochemistry of vision, in Proceedings of the Colour Vision Conference, Cambridge, England, July 28–August 3, 1947; *Documenta Ophthalmologica*, **3,** in press.

Wald, G., Interconversions of retinene and vitamin A *in vitro*, *Fed. Proc.*, 1948, **7,** 129.

Wald, G., The synthesis from vitamin A_1 of "retinene$_1$" and of a new 545 mμ chromogen yielding light-sensitive products, *J. Gen. Physiol.*, 1947–48, **31,** 489.