THE INTERCONVERSION OF THE RETINENES AND VITAMINS A *IN VITRO*

by

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In the summer of 1933 I was working as a National Research Council Fellow in Otto Meyerhof's Institute in Heidelberg, measuring the distribution of phosphates in the frog retina in light and darkness. I had noticed that the trichloracetic acid used to extract the phosphates turned the red colour of the dark adapted retina to bright orange, and that thereafter the retina behaved as a p_H indicator, orange in acid and colourless in alkaline solution. Light adapted retinas were colourless under all circumstances.

All about us the Third Reich was coming into flower, and the laboratory remained an island of sanity in a world increasingly committed to unreason and repression. Under the urging of the Society of Animal Friends, led by a retired general, the government of Baden had forbidden the killing of frogs — that is, German frogs; there seemed to be no objection to importing foreign frogs for laboratory use.

In August, just after Professor MEYERHOF and his assistants left on their vacations, and I had all but terminated my phosphate experiments, a large shipment of frogs arrived from Hungary. The *Diener* was prepared to throw them into the Neckar, but it seemed a pity to waste them, and I decided to use them to try to learn something of the orange p_H indicator which results from the destruction of rhodopsin in the retina. It was under these circumstances that I found retinene$_1$, and had a first view of its interplay with vitamin A_1 in the rhodopsin cycle.

It is only within the past few months that the chemistry of these relationships has been clarified. At a key point in this investigation it fell in with the pattern of MEYERHOF's classic experiments on the role of cozymase in the lactic fermentation. For cozymase is also the substance which reduces the retinenes to the vitamins A; and to learn this we entered on a line of experiment developed by MEYERHOF many years before.

It is therefore in a double sense that I offer this essay to OTTO MEYERHOF: first, for his personal connection with its beginnings; and again, for the debt to him and to his work which I share with all who do biochemistry.

RETINENE$_1$ AND VITAMIN A_1

Vision in dim light is mediated in all vertebrates through the retinal receptors known as rods. In land and sea vertebrates, these organs contain the red, light-sensitive

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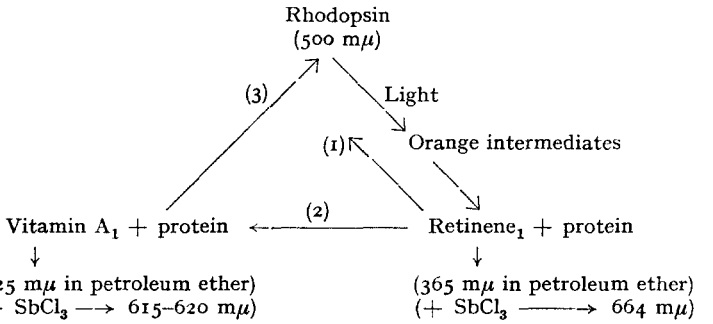
pigment rhodopsin. This substance takes part with the carotenoids retinene$_1$ and vitamin A$_1$ in a cycle of reactions of the following form*:



Rhodopsin bleaches in the light over unstable orange intermediates to a mixture of yellow retinene$_1$ and colourless protein; the retinene$_1$ is then transformed to colourless vitamin A$_1$; and both vitamin A$_1$ and retinene$_1$—or its orange precursors—recombine with protein to form new rhodopsin (WALD, 1935–36 a, b).

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

When the system is further reduced by bringing rhodopsin into aqueous solution, processes (1) and (2) are usually also eliminated. Nothing then remains but the succession of light and "dark" reactions which transform rhodopsin into retinene$_1$ and protein.

The present paper is concerned primarily with reaction (2), the conversion of retinene$_1$ to vitamin A$_1$. This is a slow, irreversible process which goes to completion in the isolated retina in about an hour at room temperature**. In 1942–43 we succeeded in bringing this process into a cell-free brei prepared from cattle retinas; and recently BLISS (1948) has shown that it occurs under certain conditions in freshly prepared rhodopsin solutions. These demonstrations that it can proceed *in vitro* form a prelude to the present experiments. Their other antecedent is the clarification of chemical relations between retinene$_1$ and vitamin A$_1$, due primarily to the work of MORTON and his colleagues in Liverpool.

Vitamin A$_1$ is the primary alcohol $C_{19}H_{27}CH_2OH$. BALL, GOODWIN, AND MORTON (1948) found that on mild oxidation this is transformed to a product which agrees in spectrum and antimony chloride reaction with retinene$_1$. They have crystallized this product and shown it to be an aldehyde, which they believe to be simply vitamin A$_1$ aldehyde, $C_{19}H_{27}CHO$. Their analytic data do not establish this formulation unequivocally as yet; but all that is now known of retinene$_1$ from the work of MORTON'S laboratory and our own is consistent with the view that it is vitamin A$_1$ aldehyde. We shall accept this as its structure in what follows.

* The wavelength values written below components of this cycle represent maxima in the absorption spectra of these substances in solution, or, when so indicated, of the products which these substances yield when treated with antimony trichloride.

** Designating this as an irreversible process is not intended to exclude the possibility that it is in fact reversible, but with the equilibrium far over toward vitamin A formation. It might for example be possible by greatly increasing the concentration of vitamin A$_1$ in the system to demonstrate a small reversion to retinene$_1$.

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THE OXIDATION OF VITAMIN A_1 TO RETINENE$_1$

In their simplest procedure for oxidizing vitamin A_1 to retinene$_1$, BALL *et al.* (1946) added a little manganese dioxide powder to a solution of vitamin A_1 in petroleum ether, and placed this mixture in a refrigerator. After 3–4 days they found that retinene$_1$ had replaced vitamin A_1 in the supernatant solution.

On examining this process we found its mechanism to be as follows. Vitamin A_1 is strongly adsorbed on manganese dioxide, and is oxidized to retinene$_1$ in the adsorbed condition. Retinene$_1$ is much less strongly adsorbed and so is displaced from the manganese dioxide by new vitamin A_1 as fast as it is formed. In this way all the vitamin A_1 passes over the manganese dioxide surface, and is replaced by retinene$_1$ in the supernatant solution. At the close of the process, the final charge of vitamin A_1 on the adsorbent is oxidized to retinene$_1$, and then, with no vitamin A_1 remaining to displace it, is oxidized further to what I have called the 545 mμ-chromogen. This can be recovered from the manganese dioxide by elution with a polar organic solvent such as ethanol.

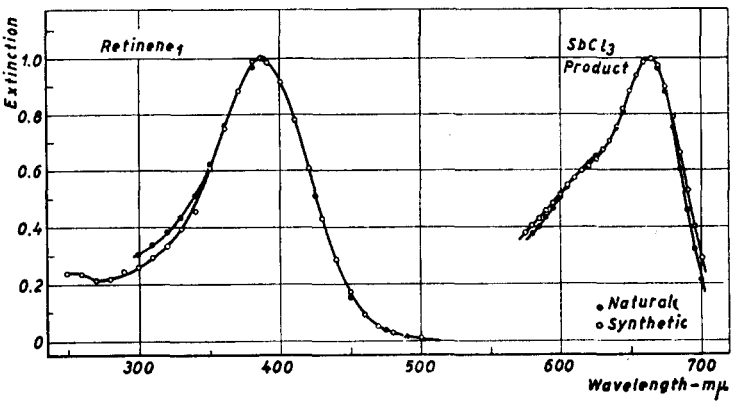

Fig. 1. Comparison of natural and synthetic retinene$_1$. Absorption spectra of cattle retinene$_1$ in chloroform and of the blue product which squid retinene$_1$ yields with antimony chloride, compared with similar preparations of synthetic retinene$_1$. The absorption is plotted as extinction or optical density, log I_o/I, in which I_o is the incident and I the transmitted intensity (From WALD, 1947–48).

For this reason the proportions of vitamin A_1 and manganese dioxide used in the procedure are important. If too much manganese dioxide is used, it adsorbs all the vitamin A_1 at once, and oxidizes all of it to the 545 mμ-chromogen (WALD, 1947–48).

Once the nature of this reaction was appreciated, we recast it in more convenient form. The manganese dioxide powder is packed into a short column such as is used in chromatography. To oxidize 10 mg of vitamin A_1, about 0.6 g of manganese dioxide is employed. A solution of crystalline vitamin A_1 in petroleum ether is poured in at the top of the column, and a solution of nearly pure retinene$_1$ is drawn off under light suction in the filtrate.

On washing through the column for a time with more petroleum ether, a high yield of retinene$_1$ is obtained. This can be freed of traces of contaminating substances by chromatographic adsorption on a column of calcium carbonate. It is adsorbed as a diffuse yellow zone, which travels slowly down the column on washing with petroleum ether, and is collected as an isolated fraction of high purity in the filtrate. The properties of this product are virtually identical with those of purified natural retinene$_1$ (Fig. 1).

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I have referred to this procedure as a *chromatographic oxidation.* The founder of chromatography, MICHAEL TSWETT, looked forward to the discovery of an entire class of such reactions, in which dry powders act at once as adsorbents and reagents, and I have no doubt that this is a correct view. Such reactions probably possess a degree of specificity and orientation not commonly realized in free solution, mimicking on occasion the character of enzymic processes. The range and properties of such chromatographic procedures deserve careful systematic examination.

THE COENZYME OF RETINENE REDUCTION*

A simple procedure has been described for oxidizing vitamin A_1 to retinene$_1$. In the retina just the reverse process occurs: retinene$_1$ is reduced irreversibly to vitamin A_1.

Several years ago, as noted above, we brought this reaction into a cell-free preparation from cattle retinas. The retinas were frozen-dried, ground to a fine powder, and were extracted exhaustively with petroleum ether, all in darkness. The residue was stirred into a brei with neutral phosphate buffer. On exposing this to light, its rhodopsin was bleached, and the retinene$_1$ so formed was converted almost completely to vitamin A_1.

In a study of the bleaching of rhodopsin in aqueous solution some years ago, we found that freshly prepared solutions undergo a special type of bleaching, which continues further than the bleaching of the same solutions after a period of aging (WALD, 1937–38). BLISS (1948) has lately reported that the basis of this extra bleaching in fresh rhodopsin solutions is the conversion of retinene$_1$ to vitamin A_1. We have confirmed this observation. A fresh rhodopsin solution, however, is not a satisfactory preparation in which to study the reduction of retinene$_1$, for while this reaction is in progress, the enzyme system which accomplishes it is being rapidly inactivated, the vitamin A_1 formed is being destroyed, and the intrusion of intermediates between rhodopsin and retinene$_1$ leaves equivocal the actual substrate in the process.

In order to analyse such systems further one would ordinarily attempt to fractionate them into their components. We had already begun such experiments when the investigation took a new turn with the discovery that the enzyme system can be fractionated anatomically through the structure of the retinal rods.

The vertebrate rod is composed of two sections, the inner and outer limbs or segments. The inner limb contains the nucleus, and is the principal seat of the ordinary cellular functions. The outer limb is a specialized outgrowth, which contains all the rhodopsin of the retina, and includes within its small compass the whole photoreceptor process.

When a retina is removed from the eye into Ringer solution with all possible care, the solution is found to contain large numbers of rod outer limbs which had broken off in the course of the dissection, just at their junctures with the inner segments. By scraping, one can break away about half the outer limbs from the surface of the retina, and collect them in a dense suspension, free from other retinal tissues, by filtration or differential centrifugation (Fig. 2).

When this procedure is carried out in dim red light, the outer limbs contain a large quantity of rhodopsin. On exposure to white light this bleaches; but in the isolated

* A detailed account of the experiments reviewed in this section will be found in the paper of WALD AND HUBBARD (1948–49).

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outer segment, unlike the whole retina, the retinene$_1$ which results is not converted to vitamin A_1. The isolated outer limb lacks some component of the system which performs this conversion.

It does not help this situation to suspend outer limbs in the presence of intact retinas. But if whole retinas are ground up in Ringer solution or phosphate buffer, though in the process almost all the outer segments are detached from other structures, the suspension which results does convert its retinene$_1$ efficiently to vitamin A_1. The crushing of the retinal cells releases substances which promote this process in the outer limbs.

If such a retinal brei is centrifuged at high speed and the clear, colourless supernatant solution is poured off, the solid residue — which retains all the rhodopsin — has lost the power to reduce retinene$_1$. It regains this capacity on re-adding to it the supernatant. Furthermore, if one suspends isolated rod outer segments in such a water extract of crushed retinas, they now reduce their retinene$_1$ to vitamin A_1. The retinal extract supplies whatever the isolated outer limb lacks for performing this conversion (Fig. 3).

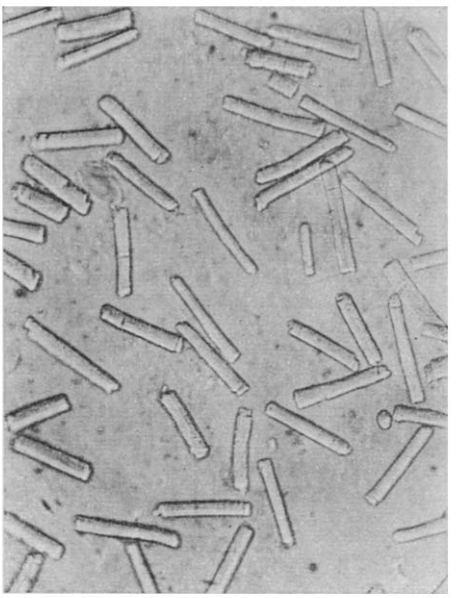

Fig. 2. Rod outer segments of the frog, suspended in Ringer solution. Magnification about 500 diameters. The longitudinal striations which can be seen in most of the outer limbs are characteristic of fresh preparations, and probably are evidence of a fibrillar structure. Later, cross-striations appear, and eventually dominate the structure; the first of these also are visible in the photograph (From WALD AND HUBBARD, 1948–49).

The water-soluble factor concerned with this process did not seem to involve a protein. It was relatively heat-stable, retaining most of its activity after boiling for as long as seven minutes. Also the ease and completeness with which it left the retinal tissue in a single extraction suggested that it was made up of small and relatively simple molecules — perhaps a coenzyme, or a hydrogen-donating substrate.

Now one would expect an enzyme protein to be relatively specific; and since retinene$_1$ is found only in retinas,

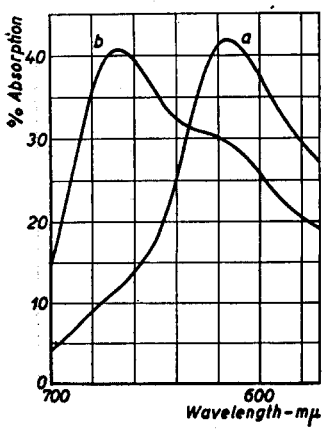

Fig. 3. Rod outer limbs suspended in a water extract of retina convert retinene$_1$ to vitamin A_1; washed retinal tissue is inactive. Isolated rod outer limbs were frozen-dried and preextracted with petroleum ether in the dark. Whole retinal tissue was ground, extracted with neutral phosphate buffer, and the outer limb material was suspended in the extract. Both this suspension and the washed retinal tissue were irradiated, incubated, and extracted with hexane. Spectra of the antimony chloride tests of these extracts are shown. That from the washed retinal tissue displays the band of unchanged retinene$_1$ (curve *b*); while the outer limb preparation suspended in retinal washings has converted its retinene$_1$ entirely to vitamin A_1 (curve *a*). (From WALD AND HUBBARD, 1948–49).

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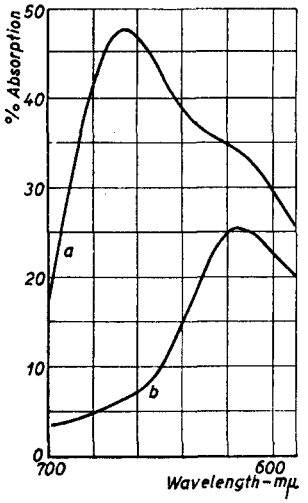

its reductase might well be restricted to this tissue. A coenzyme or substrate, however, would ordinarily be unspecific, and one would expect to find it widely distributed among the tissues. This thought led us to try an extract of frog muscle as a suspension medium for rod outer limbs.

The preparation we used was the *Muskelkochsaft* — the boiled muscle juice — of MEYERHOF (1918). Rod outer segments suspended in this medium converted their retinene$_1$ quantitatively to vitamin A$_1$ (Fig. 4).

Fig. 4. Boiled muscle juice activates isolated rod outer limbs. Equal numbers of 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. The 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. (From WALD AND HUBBARD, 1948–49).

Boiled muscle juice contains a number of substances which could donate hydrogen for the reduction of retinene$_1$. It also contains a major coenzyme of hydrogen transfer, cozymase, Coenzyme I, or DPN.

When rod outer limbs were suspended in a buffer solution to which DPN had been added, they failed to transform their retinene$_1$ to vitamin A$_1$. But if they—or an inactive preparation of washed retinal tissue—were provided with reduced cozymase, DPN–H$_2$, they performed this conversion quantitatively (Fig. 5).

Given a proper substrate, rod outer limbs can themselves reduce cozymase. We have found a first such substrate in fructose diphosphate. Rod outer segments suspended in a solution to which both DPN and fructose diphosphate were added converted their retinene$_1$ completely to vitamin A$_1$. The outer segments must therefore contain an enzyme system for reducing DPN when a suitable hydrogen donor is made available. It is highly improbable that fructose diphosphate itself is the source of hydrogen in this reaction. More probably the outer limbs also possess the enzyme aldolase, which cleaves fructose diphosphate to yield 3-glyceraldehyde phosphate, the normal substrate for the reduction of DPN in the lactic acid fermentation.

The conversion of retinene$_1$ to vitamin A$_1$ is therefore a coupled reduction in which DPN–H$_2$ acts as coenzyme. The essential process is the transfer of two

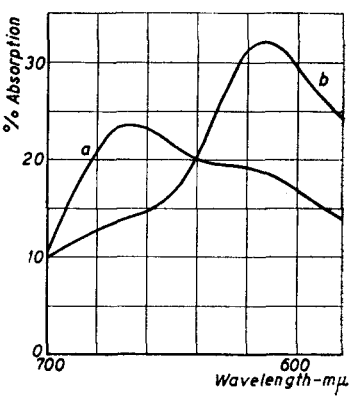

Fig. 5. The action of reduced cozymase on washed retina. Equal portions of a preparation of water-extracted frog retina were suspended in a solution containing reduced DPN, and in an otherwise identical solution lacking only the DPN–H$_2$. Both suspensions were bleached in the light, incubated, and the 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*). (From WALD AND HUBBARD, 1948–49).

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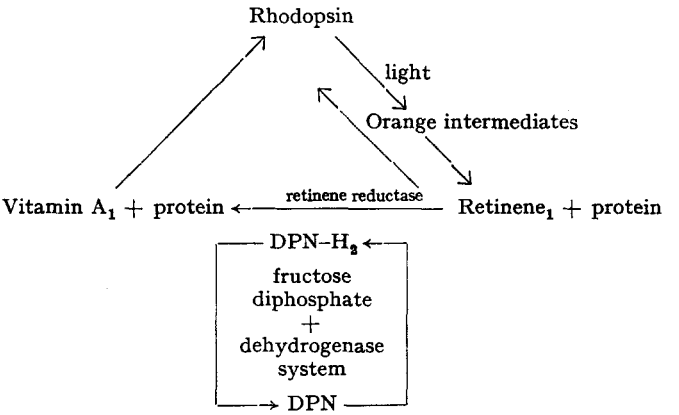
hydrogen atoms from DPN–H_2 to retinene$_1$, reducing its aldehyde group to the primary alcohol group of vitamin A_1. We may assume that in this process an apoenzyme, retinene reductase, still to be revealed, takes part. The reaction may be written:

$$\underset{\text{retinene}_1}{C_{19}H_{27}CHO} + \text{DPN–}H_2 \xrightarrow{\text{retinene reductase}} \underset{\text{vitamin } A_1}{C_{19}H_{27}CH_2OH} + \text{DPN}$$

In the rod outer limb this system works in conjunction with a second dehydrogenase system which reduces DPN, using a derivative of fructose diphosphate as hydrogen donor. The total process may be formulated:



THE RETINENE REDUCTASE SYSTEM*

With the coenzyme, the first component of the retinene reductase system was defined. Up to this point the apoenzyme had remained a matter of surmise, buried in the structure of the rod outer limb. The substrate had been obtained by bleaching rhodopsin, and was both equivocal in character and very limited in quantity.

The nature of the substrate was resolved with the observation that for this one could use pure synthetic retinene$_1$ prepared as described above by the chromatographic oxidation of crystalline vitamin A_1 on manganese dioxide. Retinene$_1$ is fat-soluble, and was originally introduced into the system with the aid of digitonin, with which it forms a water-soluble complex. Later the digitonin proved to be unnecessary, for reasons to be discussed below.

The apoenzyme was found to be readily extracted with dilute salt solutions from homogenates of frog or cattle retinas, forming clear, almost colourless solutions. Though the apoenzyme has not yet been isolated as a pure substance, it has been separated from the other components of the system and some of its properties have been determined. It is precipitated by half-saturated ammonium sulphate and re-dissolves without losing its activity. It is retained by a Visking membrane, and survives dialysis for 18 hours at 5° C against neutral phosphate buffer. It is destroyed by heating at 100° within 30 seconds. Its p_H optimum lies at about 6.5.

The retinene reductase system can therefore now be assembled from its separate components, all in true solution: the coenzyme, DPN–H_2, prepared by the method

* A short account has been published of the experiments which follow (WALD, 1949). A more complete description of these experiments will appear in the *Journal of General Physiology*.

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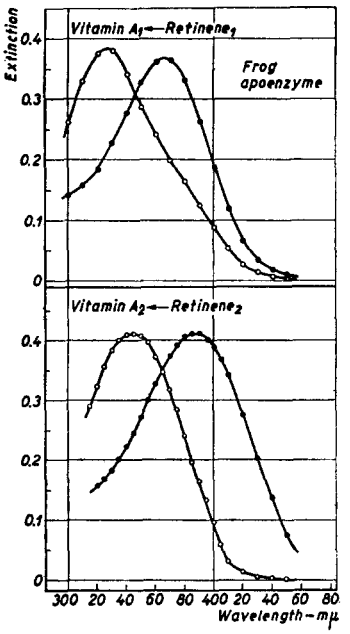
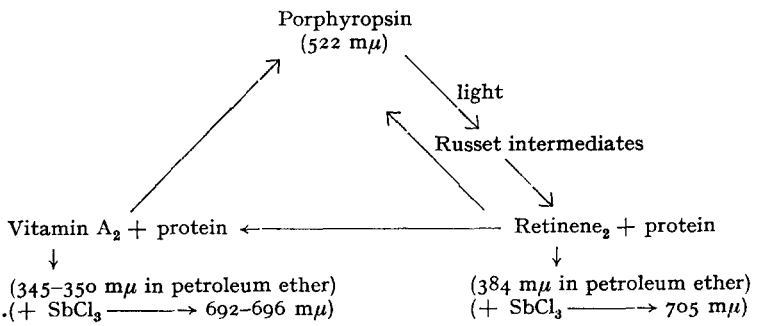


of OHLMEYER (1938); the substrate, synthetic retinene$_1$; and the apoenzyme, contained in a clear, almost colourless extract of homogenized frog or cattle retinas. When these three components are mixed and incubated for 1–2 hours at room temperature, the retinene$_1$ is quantitatively reduced to vitamin A_1 (Fig. 6, upper half).

Fig. 6. The action of frog retinene reductase on synthetic retinene$_1$ and retinene$_2$. Each of the experimental mixtures included a synthetic retinene dissolved in 1% digitonin, 0.7 mg of reduced cozymase per ml, 5.5 mg of nicotinamide per ml, and extracts of homogenized frog retinas in m/30 phosphate buffer, p_H 6.81. The controls differed only in that the retinal extracts were replaced with either the same extract which had been boiled for ½ minute (upper figure) or with the phosphate buffer alone (lower figure). The enzyme and control mixtures were incubated together for 2 hours at 23° C. Methanol was added to each to a concentration of 60%, and they were extracted with hexane. The spectra of the hexane extracts are shown. Those from the controls (solid circles) show the spectra of the unaltered retinenes; those from the enzyme mixtures (open circles) show complete conversion to the corresponding vitamins A.

RETINENE$_2$ AND VITAMIN A_2; SPECIFICITY OF RETINENE REDUCTASE

In the rods of freshwater fishes, cyclostomes and certain amphibia, rhodopsin is replaced by the purple, light-sensitive porphyropsin. This takes part in a retinal cycle identical in form with the rhodopsin system, but based upon the new carotenoids, retinene$_2$ and vitamin A_2 (WALD, 1937; 1945–46):



The structure of vitamin A_2 is still uncertain. It seems clear, however, that like A_1 it is a primary alcohol; and that retinene$_2$, as emerges from experiments of MORTON *et al.* and from those discussed below, is in all probability its aldehyde.

MORTON, SALAH, AND STUBBS (1946) reported that when solutions of vitamin A_2 in petroleum ether are let stand in the cold over solid manganese dioxide, the vitamin is replaced by a product resembling retinene$_2$ in spectrum and antimony chloride reaction. They found that this product forms, as does retinene$_1$, a 2–4-dinitrophenylhydrazone, indicating the presence of a carbonyl group. That this substance possesses a conjugated carbonyl group is shown also by a large displacement of its spectrum in

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ethanol as compared with hexane (cf. WALD, 1947–48). That the carbonyl group replaces the primary alcohol group of vitamin A_2 is shown by the fact that though the vitamin is hypophasic, its oxidation product is epiphasic in partition between hexane and 90% methanol. This information, together with what follows, leaves little doubt that this product is retinene$_2$, and that it is the aldehyde of vitamin A_2.

As in the manufacture of retinene$_1$, we have found that the oxidation of vitamin A_2 to retinene$_2$ can be carried out conveniently in chromatographic form. The procedure is identical with that used in making retinene$_1$; but in this case only about half as much manganese dioxide is employed — 0.3 g to oxidize 10 mg of vitamin A_2. The yield of retinene$_2$ is in the neighbourhood of 50%; and it can be brought to a state of high purity by chromatographic adsorption on a column of calcium carbonate.

In our past experience one of the most remarkable properties of the porphyropsin system has been its detailed parallelism in chemical behaviour with the rhodopsin cycle. In the present instance this parallelism is maintained, for retinene$_2$ is reduced to vitamin A_2 by an enzyme system entirely similar to that which reduces retinene$_1$.

This system can be assembled from the following components: the coenzyme, DPN–H_2; the substrate, synthetic retinene$_2$, prepared by the chromatographic oxidation of vitamin A_2 on manganese dioxide; and the apoenzyme, contained in a clear, almost colourless saline extract of homogenized freshwater fish retinas (yellow perch, sunfish). When these three components are mixed and left at room temperature for two hours, the retinene$_2$ is reduced almost entirely to vitamin A_2 (Fig. 7, upper half).

Since the coenzyme of retinene reduction is common to the rhodopsin and porphyropsin cycles, one may inquire into the specificity of the apoenzyme. To test this, experiments were performed in which the frog apoenzyme was allowed to act on retinene$_2$ and the fish apoenzyme on retinene$_1$. It emerged that the reduction proceeded as smoothly and completely with the crossed as with the normal substrates (Figs 6 and 7).

There is no reason therefore to designate the apoenzyme differently in the rhodopsin and porphyropsin systems. We have to deal with a single apoenzyme, retinene reductase, which with the single coenzyme, dihydrocozymase, reduces either of the retinenes to the corresponding vitamin A.

This enzyme system introduces a new vitamin into the chemistry of rod vision, for the central component

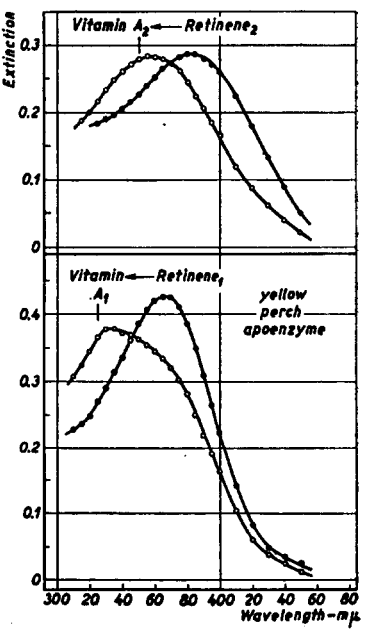

Fig. 7. Action of retinene reductase from a freshwater fish on synthetic retinene$_2$ and retinene$_1$. The experimental mixtures included solutions of the retinenes in 1% digitonin, 2.4 mg reduced cozymase per ml, 6–7 mg nicotinamide and 1 mg α-tocopheryl phosphate per ml to stabilize the system; and extracts of homogenized yellow perch retinas in m/30 phosphate buffer, p_H 6.81. The controls differed only in that the retinal extracts were replaced by the phosphate buffer alone. All the mixtures were left for 2 hours at 22° C; then methanol was added to a concentration of 60%, and they were extracted with hexane. The spectra of the hexane extracts are shown. Those from the controls (solid circles) show the unaltered retinenes; those from the enzyme mixtures (open circles) show almost complete conversion to the corresponding vitamins A. In each figure a short vertical line shows the position of the absorption maximum of vitamin A_2 or A_1 in hexane.

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of cozymase is nicotinamide, the anti-pellagra factor of the vitamin B complex. It presents also the novel phenomenon of widely distinct vitamins not only interacting *in vitro*, but of one of them participating directly in the regeneration of the others. I do not know a comparable relation in the whole of biochemistry.

STABILITY

It has been known for some time that animal and certain plant tissues contain a nucleotidase which cleaves cozymase and dihydrocozymase, and which is released into homogenates and tissue breis by the breaking of the cells. Measurements made on various tissues of the rat have shown this enzyme to be particularly active in brain, to which of course retina is closely related (MANN AND QUASTEL, 1941; HANDLER AND KLEIN, 1942). The action of this enzyme makes a number of the preparations which we have described unstable.

It was noted above that solutions of rhodopsin, prepared by extracting fresh retinal tissue with water solutions of digitonin, rapidly lose the power to reduce retinene$_1$. Within 3–4 hours their ability to perform this process usually falls to very low levels. The principal cause of this failure is the loss of cozymase.

This is shown by the following experiment. A freshly prepared rhodopsin solution was kept at about 23° C for 18 hours. At the end of this period it was divided into halves, and to one half reduced cozymase was added (1.5 mg per ml). Both portions were bleached in the light and were incubated for 1 hour. The control portion converted no more than a trace of its retinene$_1$ to vitamin A$_1$; that to which DPN–H$_2$ was added had completed this conversion. It is clear that the apoenzyme in such preparations is relatively stable; their loss of activity is caused by the destruction of the coenzyme.

Cozymase and reduced cozymase are protected from the action of the nucleotidase by the presence of free nicotinamide (2–20 mg per ml) (MANN AND QUASTEL, 1941; HANDLER AND KLEIN, 1942). It has recently been reported also that α-tocopheryl phosphate (about 1 mg per ml) similarly protects cozymase (SPAULDING AND GRAHAM, 1947).

The nucleotidase has been reported to be in general insoluble in water or dilute salt solutions. Our experiments show that it does go into solution in the 2% digitonin with which we extract rhodopsin. It also is active in all our retinal homogenates and particulate preparations. Whether it enters the saline extracts which contain our apoenzyme we have not yet determined. A number of our fish enzyme preparations have definitely been unstable, but they also tend to be slightly turbid, and may contain small amounts of very fine particles.

In any case we have taken the precaution ordinarily to add nicotinamide to our enzyme preparations; and to those from freshwater fish retinas, in which the nucleotidase appears to be particularly active, we have added also α-tocopheryl phosphate.

These adjustments extend still further the participation of vitamins in the retinene reductase system. Nicotinamide acts not only as the key component of the cozymase molecule, but in the free condition protects cozymase from destruction. In this action it is aided by vitamin E phosphate. As many as three vitamins therefore interact with one another in this single system.

THE STATE OF THE RETINENES

With the first use of the synthetic retinenes as substrates there arose the problem how, as typically fat-soluble substances, they were to be introduced into the aqueous

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enzyme system. This was solved initially by bringing the retinenes into water solution with the aid of digitonin, with which they form water-soluble complexes.

The use of digitonin, however, proved to be unnecessary. The retinal extracts which contain the apoenzyme take up the retinenes directly. If either of the retinenes is concentrated in a few drops of petroleum ether, and is agitated together with a water extract of retinas while the last of the petroleum ether is drawn off under suction, the retinenes gradually are taken up to yield clear yellow solutions. This is one indication that the retinenes couple with water-soluble substances from the retina. Primarily in these preparations they attach to protein, for they are precipitated from such solutions with the protein fraction.

It has been known for some time that in the product of bleaching rhodopsin *in solution*, most of the retinene$_1$ is found loosely coupled with protein (WALD, 1937–38, pp. 812–813). In this condition it behaves as a p_H indicator, deep yellow in acid and almost colourless in alkaline solution; hence LYTHGOE's proposal that it be called "indicator yellow". Synthetic retinene$_1$ does not change its spectrum at all with p_H; nor does natural retinene$_1$ after partial purification by adsorption and elution (WALD, 1947–48). BALL *et al.* have now shown that the p_H indicator property is characteristic of retinene$_1$ in the coupled condition (BALL, COLLINS, MORTON, AND STUBBS, 1948). Retinene$_1$ condenses spontaneously, as do aldehydes generally, with a variety of amino compounds — proteins, amino acids, aromatic amines — and in this state acts as a p_H indicator. Indeed a second evidence that the synthetic retinenes added directly or in digitonin solution to our apoenzyme extracts couple with other molecules is that they have acquired this property. They have in fact come to resemble closely the natural products of bleaching rhodopsin and porphyropsin in solution.

A third evidence that synthetic retinene$_1$ couples with other molecules in our enzyme system is that it becomes more and more difficult to extract with fat solvents as the mixture is made more alkaline. If to a solution of retinene$_1$ in digitonin one adds methanol in a final concentration of 60% and shakes vigorously with petroleum ether, almost all the retinene enters the petroleum ether regardless of the p_H. But if retinene$_1$ in digitonin is mixed with a water extract of the retina prior to carrying out this procedure, smaller and smaller fractions of the retinene enter the petroleum ether as the alkalinity is increased. At p_H 4 about 2/3 of the retinene is extracted with petroleum ether in one partition; at p_H 9 only about 1/6 of the retinene is extracted. What this probably means is that since retinene$_1$ is coupled by the condensation of its carbonyl group with the amino groups of other molecules, alkalinity favours this process by increasing the proportion of free amino groups, while acidity hinders it by converting amino groups to ammonium ions*.

The net result of these considerations is that we must regard the normal state of the retinenes in retinas and retinal extracts as a labile equilibrium between free molecules and those loosely coupled to other substances. There is no unique retinal molecule, however, with which the retinenes couple and which therefore should be designated "visual yellow" or "indicator yellow". On the contrary, the retinenes regularly condense with a variety of molecules, some protein, some forming fat-soluble complexes. So, for example, when the retinenes have been extracted from retinas with petroleum ether,

* On observing that retinene$_1$ is not readily extracted with petroleum ether from alkaline solutions of bleached rhodopsin, BLISS (1948) concluded that it had not been formed. It is formed, but like added retinene$_1$ it is retained by coupling with other retinal molecules.

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and are hence protein-free, they still behave as p_H indicators, and are therefore still in the coupled condition.

Not only do the retinenes form a variety of retinal complexes, but normally they migrate from one such association to another. One such migration is established by the present experiments. Rhodopsin and retinene reductase are different proteins. Retinene$_1$ originates on rhodopsin protein, but it must transfer to the reductase protein preparatory to its reduction. Retinene$_2$ is involved in a like situation. Such changes of the molecules with which the retinenes are coupled must play an important part in retinal metabolism.

SUMMARY

The retinene$_1$ which results from the bleaching of rhodopsin now appears to be vitamin A_1 aldehyde. MORTON *et al.* have given the best evidence for this, and have shown that retinene$_1$ can be prepared by the mild oxidation of vitamin A_1. A simple procedure is described for performing this process chromatographically on a column of manganese dioxide.

In the retina, retinene$_1$ is converted irreversibly to vitamin A_1 by an enzyme system in which reduced cozymase (reduced Coenzyme I, DPN–H_2) serves as coenzyme. The essential process is the transfer of two hydrogen atoms from DPN–H_2 to retinene$_1$, reducing its aldehyde group to the primary alcohol group of vitamin A_1.

The enzyme system which performs this reduction can be assembled in solution from the following components: the coenzyme, DPN–H_2; as substrate, synthetic retinene$_1$; and the apoenzyme extracted with dilute salt solutions from homogenized frog or cattle retinas. The apoenzyme is non-dialysable, is precipitated by half-saturated ammonium sulphate, and is destroyed by heating at 100° C within 30 seconds. Its p_H optimum lies at about 6.5.

In the rods of freshwater fishes, a parallel enzyme system reduces retinene$_2$ to vitamin A_2. This can be assembled from the following components, all in true solution: the coenzyme, DPN–H_2; as substrate, synthetic retinene$_2$, prepared by the chromatographic oxidation of vitamin A_2 on manganese dioxide; and the apoenzyme extracted with dilute salt solutions from freshwater fish retinas (sunfish, yellow perch).

The apoenzyme from frog retinas reduces retinene$_2$ as effectively as retinene$_1$. Similarly the fish apoenzyme acts equally well upon both retinenes. One need consider only one apoenzyme, retinene reductase, which together with one coenzyme, DPN–H_2, reduces either of the retinenes to the corresponding vitamin A.

The retinene reductase system brings a second vitamin into the chemistry of rod vision. It presents the novel phenomenon of one vitamin regenerating another, for the central component of DPN–H_2 is nicotinamide, the anti-pellagra factor of the vitamin B complex.

Rhodopsin solutions and retinal homogenates rapidly lose their power to reduce the retinenes, through destruction of their DPN by a nucleotidase. Rhodopsin solutions which have lost their activity in this way are re-activated by the addition of new DPN–H_2. The coenzyme can also be protected by the presence of free nicotinamide and of α-tocopheryl phosphate.

On addition to the enzyme system, the synthetic retinenes rapidly couple with other molecules, and primarily with protein. The normal state of the retinenes in retinas and retinal extracts is a labile equilibrium between the free and the coupled condition. The retinenes couple with a variety of retinal molecules, and migrate freely from one to the other.

RÉSUMÉ

Le rétinène$_1$, qui résulte du blanchissement de la rhodopsine, apparaît maintenant comme étant l'aldéhyde de la vitamine A_1. MORTON *et collab.* en ont donné la meilleure preuve en montrant que le rétinène$_1$ peut être préparé par une oxydation ménagée de la vitamine A_1. Un procédé simple est décrit, qui permet d'effectuer cette opération par chromatographie sur une colonne de bioxyde de manganèse.

Dans la rétine, le rétinène$_1$ est converti irréversiblement en vitamine A_1 par un système enzymatique dans lequel la cozymase I réduite (DPN–H_2) sert de coenzyme. Le processus consiste essentiellement en un transfert de deux atomes d'hydrogène du DPN–H_2 sur le rétinène$_1$, réduisant sa fonction aldéhydique en fonction alcoolique primaire de la vitamine A_1.

Le système enzymatique qui effectue cette réduction peut être constitué en solution à partir

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des composantes suivantes: la coenzyme, DPN–H_2; comme substratum du rétinène$_1$ synthétique; et l'apoenzyme, extraite de rétines homogénéisées de grenouilles ou de bœufs au moyen de solutions salines diluées. L'apoenzyme n'est pas dialysable; elle est précipitée par le sulfate d'ammonium à demi-saturation et détruite par chauffage à 100° pendant 30 secondes. Son p_H optimum est d'environ 6.5.

Dans les bâtonnets de la rétine de poissons d'eau douce, il existe un système enzymatique parallèle, qui réduit le rétinène$_2$ en vitamine A_2. Ce système peut être constitué à partir des composantes suivantes, toutes en vraie solution: la coenzyme, DPN–H_2; comme substratum, du rétinène$_2$ synthétique, préparé par oxydation chromatographique de la vitamine A_2 au bioxyde de manganèse; et l'apoenzyme, extraite au moyen de solutions salines diluées à partir de rétines homogénéisées de poissons d'eau douce (poisson-soleil, perche jaune).

L'apoenzyme de la rétine de grenouille réduit le rétinène$_2$ aussi bien que le rétinène$_1$. De même, l'apoenzyme de poissons d'eau douce agit également bien sur les deux rétinènes. Il n'est donc besoin de considérer qu'une seule apoenzyme, la réductase du rétinène, qui, en présence d'une coenzyme, le DPN–H_2, réduit l'un ou l'autre des deux rétinènes en la vitamine A correspondante.

Le système de la réductase du rétinène introduit une seconde vitamine dans la chimie de la vision par bâtonnets. Il présente le phénomène nouveau d'une vitamine qui en régénère une autre, attendu que la composante essentielle du DPN–H_2 est la nicotamide, le facteur antipellagreux du complexe vitamique B.

Des solutions de rhodopsine et d'extraits homogénéisés de rétines perdent rapidement leur pouvoir de réduire les rétinènes, de par la destruction de leur DPN par une nucléotidase. Des solutions de rhodopsine ayant ainsi perdu leur pouvoir réducteur sont réactivées par l'addition d'une quantité fraîche de DPN–H_2. La coenzyme peut également être protégée par la présence de nicotamide libre et de phosphate d'α-tocophéryle.

En plus du système enzymatique étudié, les rétinènes synthétiques forment des produits d'addition avec d'autres molécules, et spécialement avec les protéines. L'état normal des rétinènes dans les rétines et leurs extraits est un équilibre labile entre la forme libre et la forme associée. Les rétinènes s'associent avec une variété de molécules rétinales et migrent librement de l'une à l'autre.

ZUSAMMENFASSUNG

Das Retinen$_1$, welches bei der Bleichung des Rhodopsins entsteht, entpuppt sich jetzt als Vitamin A_1-Aldehyd. MORTON *und Mitarb.* haben dafür den besten Beweis geliefert, dadurch dass sie gezeigt haben dass Retinen$_1$ durch milde Oxydation von Vitamin A_1 gebildet werden kann. Es wird eine einfache Prozedur beschrieben, um diesen Vorgang chromatographisch mittels einer Mangandioxyd-Säule zu bewerkstelligen.

In der Netzhaut wird Retinen$_1$ irreversibel in Vitamin A_1 verwandelt durch ein Enzymsystem in welchem reduzierte Cozymase I (DPN–H_2) als Coenzym dient. Die Hauptreaktion besteht dabei in der Übertragung von zwei Wasserstoffatomen vom DPN–H_2 auf das Retinen$_1$, dessen Aldehydgruppe zur primären Alkoholgruppe des Vitamins A_1 reduziert wird.

Das Enzymsystem welches diese Reduktion vollführt, kann in Lösung aus folgenden Komponenten zusammengestellt werden: das Coenzym, DPN–H_2; als Substrat, synthetisches Retinen$_1$; und das Apoenzym, welches durch verdünnte Salzlösungen aus homogenisierten Frosch- oder Rinder-Netzhäuten ausgezogen wird. Das Apoenzym ist nicht dialysierbar; es wird durch halbgesättigte Ammoniumsulfat-Lösung gefällt und durch Erhitzen auf 100° innerhalb 30 Sek. zerstört. Sein p_H Optimum liegt bei *ca* 6.5.

In den Stäbchen von Süsswasserfischen besteht ein paralleles Enzymsystem, welches Retinen$_2$ zu Vitamin A_2 reduziert. Es kann aus folgenden, alle in wahrer Lösung befindlichen Komponenten zusammengestellt werden: das Coenzym, DPN–H_2; als Substrat, synthetisches Retinen$_2$, durch chromatographische Oxydation von Vitamin A_2 an Mangandioxyd dargestellt; und das Apoenzym, welches durch verdünnte Salzlösungen aus den Netzhäuten von Süsswasserfischen (Sonnenfisch, gelber Barsch) ausgezogen wird.

Das Apoenzym aus Froschnetzhäuten reduziert Retinen$_2$ so wirksam wie Retinen$_1$. Desgleichen wirkt das Fisch-Apoenzym gleich gut an beiden Retinenen. Man hat also nur ein einziges Apoenzym zu betrachten, die Retinen-Reduktase, welche zusammen mit einem Coenzym, dem DPN–H_2, beide Retinene zu den entsprechenden A-Vitaminen reduziert.

Das System der Retinen-Reduktase führt ein zweites Vitamin in die Chemie des Stäbchen-Sehens ein. Es zeigt das neuartige Phänomen eines Vitamins welches ein anderes regeneriert, denn die wichtigste Komponente vom DPN–H_2 ist das Nikotinamid, der Antipellagra-Faktor des Vitamin B-Komplexes.

Lösungen von Rhodopsin und homogenisierten Netzhautextrakten verlieren rasch ihr Vermögen, Retinene zu reduzieren; ihr DPN wird nämlich von einer Nukleotidase zerstört. Auf solche Art inaktivierte Rhodopsin-Lösungen können durch Zugabe von DPN-H_2 reaktiviert werden. Das

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Coenzym kann auch durch die Gegenwart von freiem Nikotinamid oder von α-Tokopherylphosphat geschützt werden.

Ausser mit dem Enzymsystem, verbinden sich die synthetischen Retinene auch rasch mit anderen Molekülarten, besonders mit Proteinen. Der Normalzustand der Retinene in der Netzhaut und in Netzhautextrakten ist ein labiles Gleichgewicht zwischen freier und gebundener Substanz. Die Retinene wandern leicht von einer zur anderen der verschiedenen in der Netzhaut befindlichen Molekeln mit denen sie lose Verbindungen eingehen.

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