CIS-TRANS ISOMERS OF VITAMIN A AND RETINENE IN THE RHODOPSIN SYSTEM

By RUTH HUBBARD and GEORGE WALD*

(*From the Biological Laboratories of Harvard University, Cambridge*)

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We have recently described the synthesis of rhodopsin in a solution containing four components: vitamin A, the precursor of the rhodopsin chromophore; opsin, the protein of rhodopsin; and liver alcohol dehydrogenase and cozymase, the enzyme and coenzyme which oxidize vitamin A to retinene (Hubbard and Wald, 1951).[1]

This experiment was first performed with a fish liver oil concentrate as the source of vitamin A. When later we attempted to repeat it using *crystalline* vitamin A, almost no rhodopsin was formed.

Such differences in behavior in two samples of what is ordinarily thought of as the same substance could have only one explanation. The vitamin A molecule exists in several different *shapes*, as different geometrical or cis-trans isomers. Liver oils are known to contain a mixture of such isomers, while ordinary crystalline vitamin A is a single isomer. Carotenoids in general are converted from any single cis-trans configuration to an equilibrium mixture of stereoisomers by irradiation with light in the presence of a trace of iodine. When crystalline vitamin A was treated in this way, it became as effective a precursor of rhodopsin as the liver oil concentrate.

These experiments made it plain that the geometrical configuration of vitamin A is a dominant factor in the rhodopsin system. The present paper is concerned with the analysis of this relation.

* This research was supported in part by the Biosciences Division of the Office of Naval Research. Portions of the investigation have been reported earlier by Hubbard and Wald (1952) and Hubbard (1952). In addition to specific contributions mentioned in the text, we wish to acknowledge our general debt to the Organic Research Laboratory of Distillation Products Industries of Rochester, New York, for repeated gifts of vitamin A and retinene preparations. We wish also to express our appreciation for the patient and skillful help of Mr. Robert Gregerman in isolating single geometrical isomers of retinene.

‡ The original paper was received November 26, 1951. The date given above is that of the receipt of the revised version.

[1] Throughout this paper the terms vitamin A and retinene refer to vitamin A_1 and retinene$_1$.

Geometrical Isomers of Vitamin A and Retinene

Like other carotenoids, vitamin A exists in a number of stereoisomeric forms, differing from one another in the cis-trans orientation of groups about its double bonds. We owe most of what is known of cis-trans isomerization in the plant carotenoids to the work of Zechmeister's laboratory. In close association with these investigations, Pauling has developed the theory of the subject (general review: Zechmeister, 1944; see also Pauling, 1939; Zechmeister, LeRosen, Went, and Pauling, 1941; Zechmeister, LeRosen, Schroeder, Polgár, and Pauling, 1943; Pauling, 1949).

According to theory, in such structures as vitamin A or retinene the cis configuration can occur readily only about those double bonds adjacent to methyl groups. In any other position the cis linkage introduces steric difficulties. The possibilities for cis-trans isomerization in vitamin A are thought, therefore, to be highly restricted. Only two double bonds, those numbered *3* and *5* in Fig. 1, should readily assume the cis configuration. Hence only four stereoisomers are expected: the all-trans, *3*-cis, *5*-cis, and *3,5*-di-cis. These structures are shown in Fig. 1. Their spatial relations are represented more adequately in the retinene models of Fig. 2.

Ordinary crystalline vitamin A, as also the bulk of the synthetic vitamin A available commercially, is the all-trans isomer. An isomer which crystallizes less readily, called neovitamin A, has been isolated from fish liver oils (Robeson and Baxter, 1947) and from synthetic preparations (Cawley *et al.*, 1948); it is thought to have the *5*-cis configuration. For reasons which will become clear below, we shall refer to this isomer hereafter as neovitamin A*a*. Graham, van Dorp, and Arens (1949) have reported the synthesis of an isomer of retinene which they believe to be the *3*-cis structure.[2] These are the only isolations of single stereoisomers of vitamin A and retinene reported up to the time of the present experiments.[3]

The absorption spectra of carotenoids change in characteristic ways with changes in geometrical configuration. In general, the main absorption band of the all-trans isomer lies at the longest wave length, and has the highest molecular extinction. A single cis linkage depresses the height of this maximum, and shifts its position 4 to 6 mμ toward shorter wave lengths. Two cis linkages tend to displace the band about twice as far. Neovitamin A*a* is peculiar in this regard, for though apparently a cis isomer, its absorption maximum lies at slightly *longer* wave lengths than that of the predominant, presumably all-trans isomer.

[2] It is not possible as yet to assign specific configurations with assurance to any of the known stereoisomers of vitamin A or retinene, and all such configurations should be regarded for the present as tentative.

[3] Dalvi and Morton (1951) have described partly purified, non-crystalline preparations of natural esters of neovitamin A*a*, and neoretinene *a*.

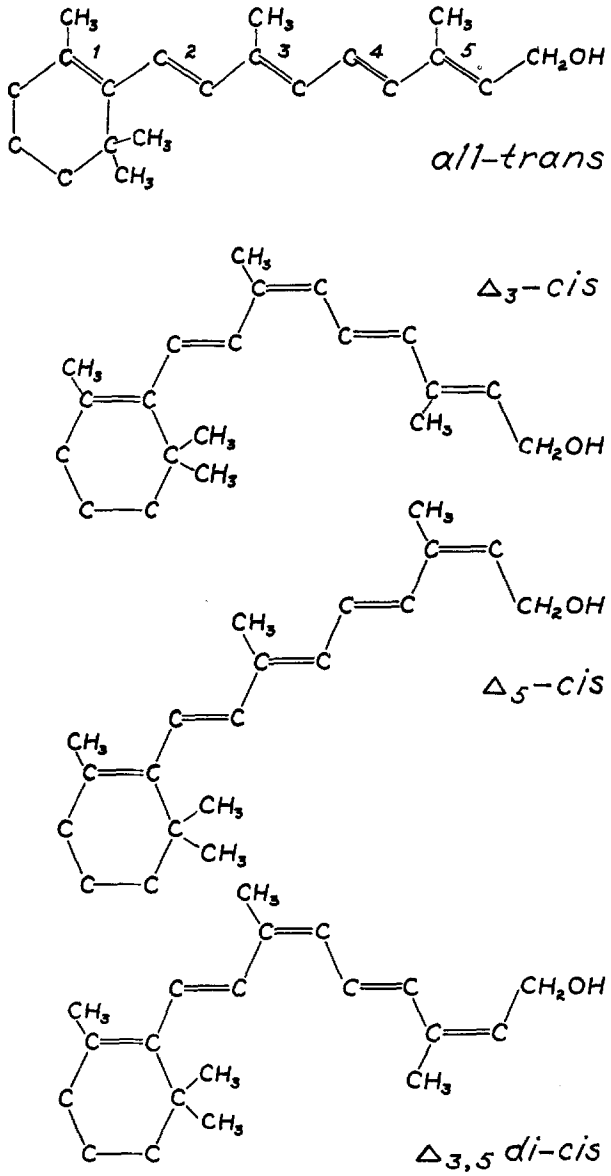

FIG. 1. Structural formulae of geometrical isomers of vitamin A. Cis-trans isomerization is expected to occur most readily about double bonds *3* and *5*. Ordinary crystalline vitamin A, as also the bulk of the commercial synthetic material, apparently is all-trans. There is little basis as yet for assigning configurations to the other isomers which have been isolated.

Fish and rat liver oils contain mixtures of vitamin A isomers.[4] Robeson and Baxter devised a simple procedure for determining the proportions of all-trans and neovitamin A*a* in liver oils, based upon the fact that maleic anhydride forms an addition complex with the former much more rapidly than with the latter. They estimate that the liver oils of a number of fishes contain 32 to 39

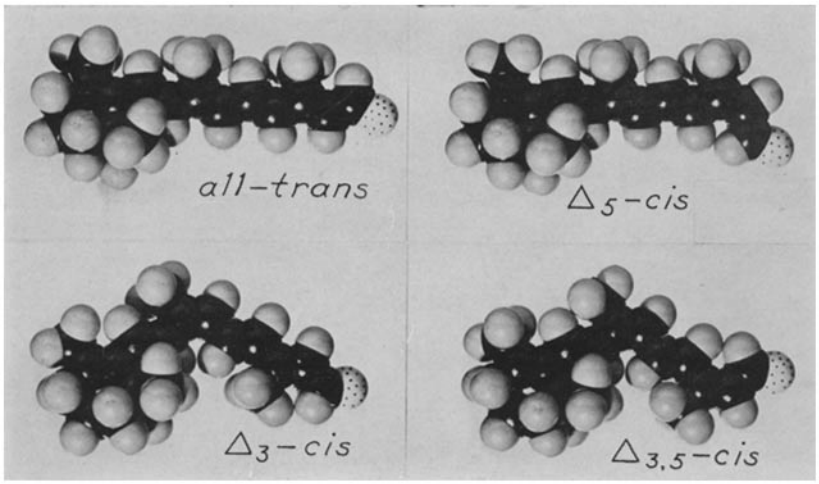

FIG. 2. Molecular models of geometrical isomers of retinene. The black segments represent carbon atoms, the white hydrogen, and the stippled oxygen. A cis linkage bends the carbon chain, but in the four configurations shown the model remains almost coplanar. A cis linkage at any other double bond than *3* or *5* not only bends but twists the carbon chain; the model, if it can be made at all, is far from coplanar. Such configurations are believed to be highly improbable, since the large departure from coplanarity should represent a break in conjugation and a consequent loss of resonance stabilization.

per cent of neovitamin A*a*; and this result has since been confirmed and extended by Meunier and Jouanneteau (1948).[5]

[4] The vitamin A of fish liver oils and concentrates is often spoken of as "natural," and considerable stress at times laid upon its supposed naturalness. Fairly drastic procedures are used, however, in making such preparations, involving long heating, exposure to light, and other circumstances which favor isomerization. There may be little relation between the proportions of geometrical isomers of vitamin A in commercial fish liver oils and those which occur *in vivo*.

[5] This reaction occurs most readily when the groups about double bond *5* are in trans configuration. In fact, therefore, it tends to divide the all-trans and *3*-cis isomers, which react rapidly, from the *5*-cis and *3,5*-di-cis isomers, which react with difficulty. Since the first member of each of these pairs predominates, however, the test to a first approximation distinguishes all-trans from *5*-cis vitamin A.

The conditions under which carotenoids stereoisomerize have been reviewed by Zechmeister (1944). A single geometrical isomer, on warming, treatment with acids, or irradiation, particularly in the presence of a trace of iodine, yields a mixture of isomers, varying in proportion with the method of isomerization, the solvent, and other conditions.[6]

Vitamin A isomerizes also in the body. Robeson and Baxter (1947) found that when rats previously depleted of vitamin A are fed either all-trans or neovitamin A*a*, both isomers are deposited in characteristic proportions in the liver. The rate at which the vitamin isomerizes *in vivo* is not known, but it seems at least to keep pace with such long term processes as growth. Presumably for this reason all-trans vitamin A, neovitamin A*a* (Robeson and Baxter, 1947), and the cis isomer of retinene prepared by Graham *et al.* (1949) all yield comparable bioassays in growth tests on the rat. Configuration does make some difference in this animal, however, for on reevaluating the results of 7 years of bioassay in their laboratory, Harris *et al.* (1951) conclude that neovitamin A*a* is 80.7 per cent as effective as the all-trans isomer in stimulating growth, and 71.5 per cent as effective in causing the deposition of vitamin A in the liver.

The particular importance of geometrical isomerism is that it involves large changes in the *shape* of the molecule. The all-trans isomer is relatively straight; the *5*-cis configuration has a kink at the end; the *3*-cis isomer is bent in the middle; and the di-cis structure is zigzag. Such differences might be of little consequence in some connections; but for the reaction with an enzyme, or indeed with any other type of protein, shape is all important. The rhodopsin system for the first time exhibits vitamin A and retinene in reaction with specific proteins. The appearance of stereoisomeric effects under these circumstances was more or less inevitable.

[6] Iodine makes two kinds of contribution to the stereoisomerization of vitamin A. On the one hand it is a general catalyst of this type of process; on the other, it sensitizes the process to visible light. On activation by light the iodine molecule—or perhaps an iodine atom resulting from its dissociation—adds to a double bond, converting it momentarily into a single bond capable of some degree of free rotation. A moment later the iodine is away again, and the double bond is reconstituted. But having been in free rotation it re-forms in either the cis or trans configuration. The second feature of this process is that the light involved is that absorbed, not by the carotenoid, but by iodine. Vitamin A can probably be isomerized by light alone, but this would require light which it absorbs; *i.e.*, ultraviolet light. Iodine, which has of course a strong visible absorption, brings this action into the visible region. On absorbing light of wave lengths shorter than 499.5 mμ, the iodine molecule dissociates into one normal and one excited atom (J. Franck; *cf.* Griffith and McKeown, 1929, p. 217). Does iodine catalyze stereoisomerization at longer wave lengths? The answer to this question should decide whether one needs only to excite the iodine molecule with light or to dissociate it.

Cis-Trans Isomers of Vitamin A in the Synthesis of Rhodopsin

In the light of the foregoing discussion, our initial observation that rhodopsin is synthesized in solution from liver oil vitamin A though not from the crystalline vitamin, means that rhodopsin is not made from the all-trans isomer, but requires instead a cis isomer present in liver oil.

The phenomenon itself is shown in Fig. 3. Our four component system was assembled in this case with cattle opsin, crystalline alcohol dehydrogenase, cozymase, and a fixed quantity of vitamin A, either crystalline or concentrated from liver oil. At the left in Fig. 3 are shown the changes in extinction at 500 mμ—the absorption maximum of rhodopsin—when these mixtures were incubated in the dark.[7] The curves at the right show the difference spectra of the final products—their differences in absorption before and after bleaching with light. All the materials and procedures that enter this and later experiments are described in the Appendix.

In this experiment liver oil vitamin A yielded a rapid and persistent synthesis of rhodopsin, still continuing after 3 hours. Almost the whole rise of extinction at 500 mμ during this interval was due to rhodopsin itself. This is shown by the very small effect of adding hydroxylamine at the end of the incubation, a reagent which breaks up fortuitous combinations of retinene with other molecules by itself appropriating the retinene to form retinene oxime; and by the difference spectrum (*A-B*) on the right, which has the form characteristic of rhodopsin.

Crystalline vitamin A, however, yielded only a trace of rhodopsin. Most of the small increase in extinction at 500 mμ to which it gave rise was spurious, and was eliminated by hydroxylamine. The difference spectra on the right show that only about one-tenth as much rhodopsin was formed from crystalline as from liver oil vitamin A.

Experiment.—Two solutions were prepared, each containing cattle opsin, about 100 μg. alcohol dehydrogenase, and 200 μg. DPN in 0.35 ml. of 1 per cent aqueous digitonin. To each mixture, 30 μg. of vitamin A was added, either as liver oil concentrate or as the crystalline substance, in each case dissolved in 0.25 ml. of 2 per cent digitonin. These mixtures were prepared in the light; the final pH was 6.9. They were both incubated in the dark, in the cell compartment of the Beckman spectrophotometer, and the rise in extinction at 500 mμ was measured periodically. This is shown at the left in Fig. 3.

After about 3 hours in the dark, 0.1 ml. of 1 M hydroxylamine was added to each sample. The absorption spectrum was measured in the dark, and again after bleaching the rhodopsin by 2 minutes' exposure to white light. The differences in absorption spectrum before and after bleaching—the "difference" spectra—are shown at the right of the figure.

[7] The extinction is the $\log_{10} I_0/I$, in which I_0 is the incident and I the transmitted intensity. This quantity is directly proportional to the concentration and the depth of layer.

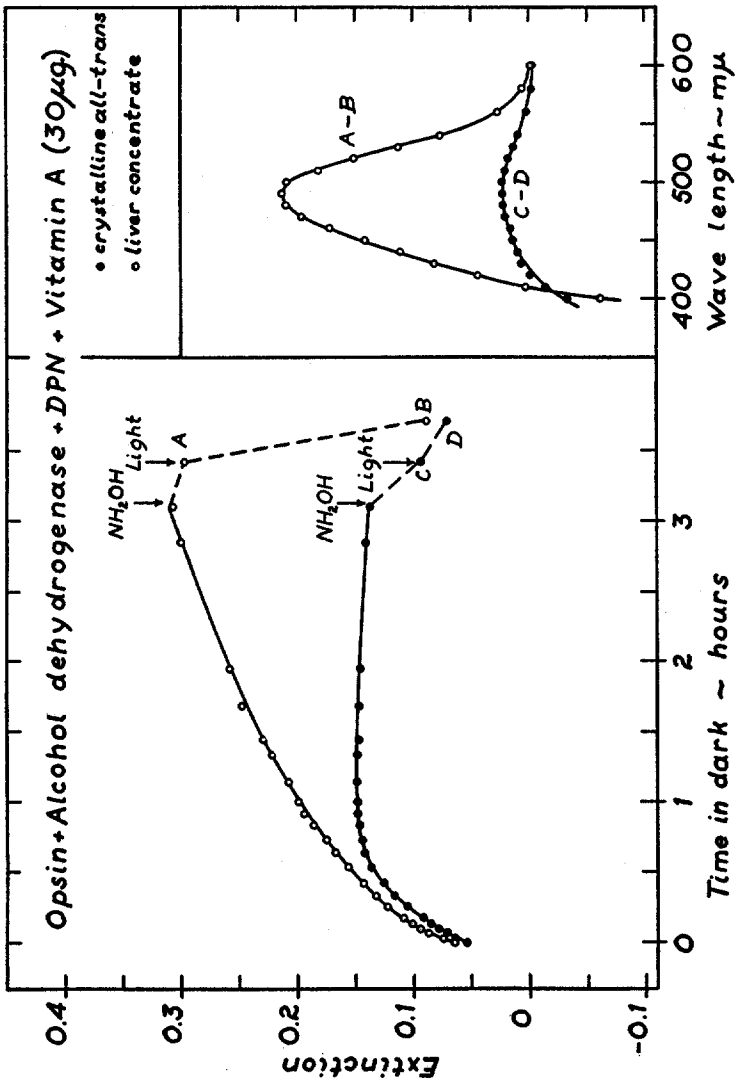

FIG. 3. The synthesis of rhodopsin from crystalline (all-trans) and from liver oil vitamin A. Cattle opsin, horse liver alcohol dehydrogenase, and cozymase (DPN) were incubated in the dark with 30 μg. vitamin A, either crystalline or the mixture of stereoisomers found in fish liver oil concentrate. At the left are shown changes in the extinction at 500 mμ, the absorption maximum of rhodopsin. After 189 minutes, hydroxylamine was added to each mixture to destroy spurious retinene complexes, and the rhodopsin which had formed was bleached with light (*A* to *B*, *C* to *D*). The differences in the absorption spectra measured before and after bleaching ("difference spectra") are shown at the right. The fish liver oil concentrate had yielded a large synthesis of rhodopsin, but the crystalline substance was relatively ineffective.

The experiment just described involved a highly purified system containing cattle opsin and horse liver alcohol dehydrogenase. Much the same results were obtained, however, with a system derived wholly from the frog retina. In this case a digitonin extract of the bleached retinas of *Rana pipiens* was prepared

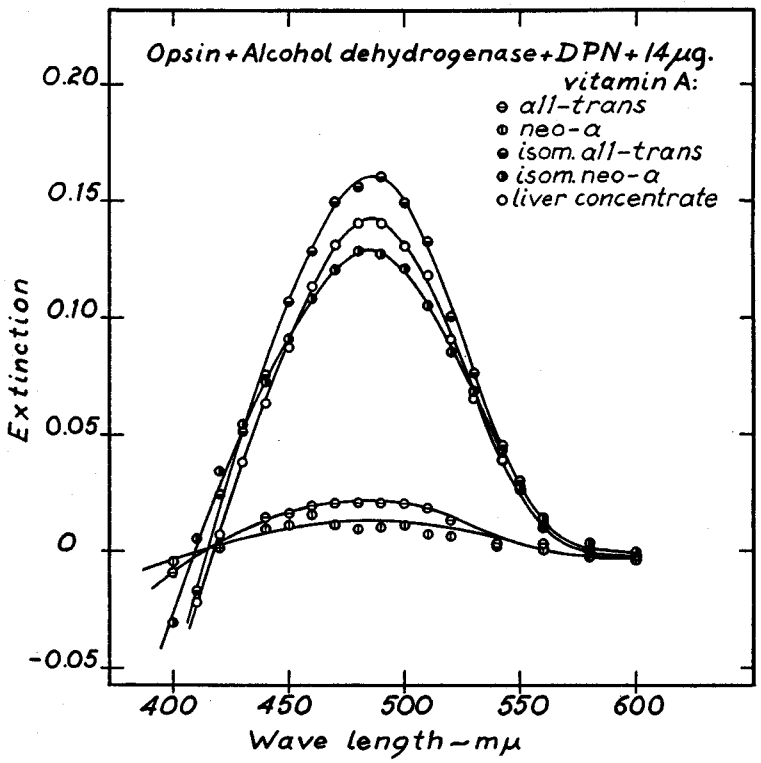

FIG. 4. The synthesis of rhodopsin from vitamin A of five different types: crystalline all-trans, crystalline neovitamin A*a*, isomerized all-trans, isomerized neo-*a*, and fish liver oil concentrate. The figure shows the difference spectra of rhodopsin obtained after incubating equal amounts of each form of vitamin A with opsin, alcohol dehydrogenase, and cozymase. All-trans vitamin A and neovitamin A*a* yielded only traces of rhodopsin; but after these substances had been isomerized with light in the presence of iodine, they were as effective as the liver oil concentrate.

as described previously (Wald and Hubbard, 1950). Such an extract contains retinene reductase (which may be identical with alcohol dehydrogenase) and opsin. Supplemented with DPN and mixed with equivalent amounts of crystalline and liver oil vitamin A, it yielded results similar to those shown in Fig. 3.

Such experiments show that rhodopsin is not synthesized from all-trans vitamin A. The crystalline neovitamin A*a* of Robeson and Baxter is equally ineffective. Both substances, however, are readily converted to an equilibrium

mixture of stereoisomers by exposure to light in the presence of a trace of iodine. After this treatment, both preparations form rhodopsin as efficiently as liver oil vitamin A. These relations are shown in Fig. 4.[8]

Experiment.—Five test mixtures were prepared, each containing the same quantity of cattle opsin, 40 μg. of alcohol dehydrogenase, and 100 μg. of cozymase, all dissolved in 0.45 ml. of 2 per cent digitonin. To each was added 14 μg of vitamin A, dissolved in 0.1 ml. of 2 per cent digitonin. The vitamin A was of five types: (*a*) crystalline all-trans; (*b*) crystalline neovitamin A*a*; (*c*) isomerized all-trans; (*d*) isomerized neovitamin A*a*; and (*e*) fish liver oil concentrate.

All these mixtures were incubated in the dark at 23°C. for 15 hours (pH 6.8), and their difference spectra measured. These are shown in the figure.

The vitamin A was isomerized as follows. A small quantity of crystalline all-trans or neovitamin A*a* (5 to 10 mg.) was dissolved in 2 ml. benzene and to this was added 0.1 ml. of a solution of iodine in benzene, containing 1 per cent as much iodine by weight as the vitamin A upon which it was to act. This mixture was illuminated for 10 minutes with the focussed white light of a 100 watt tungsten filament microscope lamp. Then it was stirred with a pinch of powdered sodium thiosulfate ($Na_2S_2O_8$) to remove residual iodine, centrifuged, the benzene evaporated off under reduced pressure, and the vitamin A taken up in 2 per cent digitonin.

In aqueous digitonin solution, the absorption maximum of all-trans vitamin A is at 330.5 mμ; on isomerization it moves upward, to 332 mμ. The absorption maximum of neovitamin A*a* is at 334 mμ; on isomerization it moves downward, also to 332 mμ. These changes are just opposite in direction to what one expects of all-trans and cis isomers. The retinenes derived from these vitamin A isomers, however, display the conventional spectral relations.

The experiment shown in Fig. 4 leaves no doubt that the differences which we have encountered in vitamin A preparations have their source in geometrical isomerism. This experiment also eliminates neovitamin A*a* as a precursor of rhodopsin. The next task would appear to be to isolate from some active preparation the specific stereoisomer which makes rhodopsin. It will next be shown, however, that this task is better undertaken with retinene than with vitamin A.

The Site of Isomer Specificity

We have already noted that such configurational specificity as we encounter here is probably associated with protein reactions. The synthesis of rhodopsin however, involves two proteins, and indeed is a two step process. In the first step the enzyme protein, alcohol dehydrogenase, catalyzes the oxidation of vitamin A to retinene. In the second step, retinene condenses with the protein opsin to form rhodopsin. Which of these reactions is isomer-specific?

[8] The same experiment was performed with a commercial preparation of synthetic vitamin A. Used directly, this yielded only a trace of rhodopsin. After isomerization by light in the presence of iodine, it yielded about five times as much rhodopsin.

To answer this question we have examined separately the reactions of alcohol dehydrogenase with all-trans and liver oil vitamin A, and the corresponding retinenes; and of opsin with the retinenes.

The first process was studied by measuring the rates of oxidation of crystalline all-trans and liver oil vitamin A, and the rates of reduction of the corresponding retinenes. These reactions together compose the following system (Wald and Hubbard, 1948–49; Wald, 1950)[9]:

$$C_{19}H_{27}CH_2OH + DPN \underset{}{\overset{\text{alcohol dehydrogenase}}{\rightleftharpoons}} C_{19}H_{27}CHO + DPN{-}H_2$$

Vitamin A + cozymase — retinene + reduced cozymase

Fig. 5 shows two experiments on the isomer specificity of alcohol dehydrogenase. The oxidation to retinene of all-trans vitamin A and liver oil vitamin A is shown at the left. In the system as we have assembled it, about twice as much retinene is formed from all-trans as from liver oil vitamin A.

The reverse reaction is shown at the right. All-trans and liver oil vitamin A had been oxidized on manganese dioxide to the corresponding forms of retinene (see Appendix), and are here being reduced back to vitamin A by the enzyme system. It appears from this experiment that the rate and extent of reduction of both samples of retinene are closely similar, yet again slightly to the advantage of the all-trans form. This result is not altogether reliable for at the time we did not yet know of the need to protect retinene from isomerization by light (see below). Some isomerization of all-trans retinene may therefore have occurred during the procedure; in this case the result shown tends to underestimate the advantage of the all-trans isomer.

Experiment.—Vitamin A, dihydrocozymase, and retinene all have absorption bands in the near ultraviolet. In digitonin solution vitamin A has an absorption maximum at about 330 mμ, retinene at 384–389 mμ, depending upon its isomeric state; and reduced DPN at 340 mμ. We chose 390 mμ as the wave length at which to follow changes in the concentration of retinene. The molar extinctions of vitamin A and reduced cozymase are such that only 8 per cent of the the total change of extinction at this wave length is caused by their participation in the reaction. To a first approximation therefore one can use the uncorrected extinction at 390 mμ as a measure of retinene concentration.

In the experiment shown at the left in Fig. 5, two portions of crystalline alcohol dehydrogenase, about 40 μg. each, were incubated with 230 μg. of DPN and 14 μg. of either all-trans or liver oil vitamin A. These materials were incubated in 2.25 ml. of 0.18 per cent aqueous digitonin, at pH 7 and 20°C. The extinction at 390 mμ, a measure of the concentration of retinene, rose rapidly in both samples and leveled off within 10 to 15 minutes. At the end of 30 minutes, about twice as much retinene had been formed from crystalline as from liver oil vitamin A.

The right half of Fig. 5 shows the reverse reaction. Two samples of alcohol dehydro-

[9] The equilibrium between vitamin A and retinene in this system as a function of hydrogen ion concentration has recently been studied by Bliss (1951).

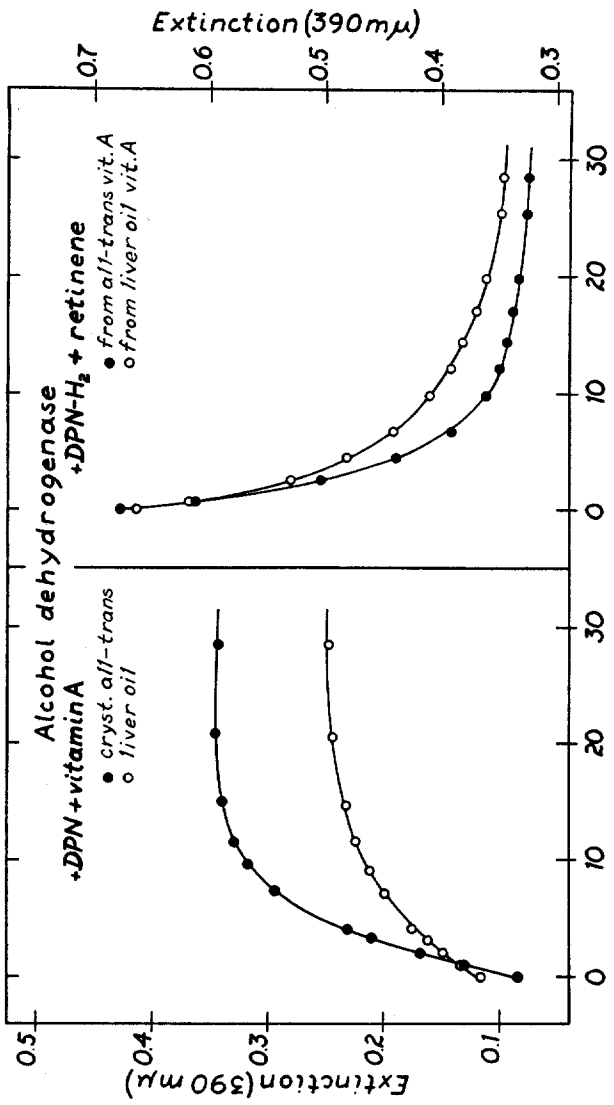

FIG. 5. The isomer specificity of liver alcohol dehydrogenase. The crystalline enzyme and its coenzyme were allowed to act upon crystalline all-trans and liver oil vitamin A and upon the corresponding retinenes. The figure shows changes in the extinction at 390 mμ, which measures the retinene concentration. At the left vitamin A is oxidized, at the right retinene is reduced, in each case to an equilibrium mixture of vitamin A and retinene. In both directions the reaction proceeds rapidly with both forms of the substrate, with a small advantage in favor of the all-trans isomer.

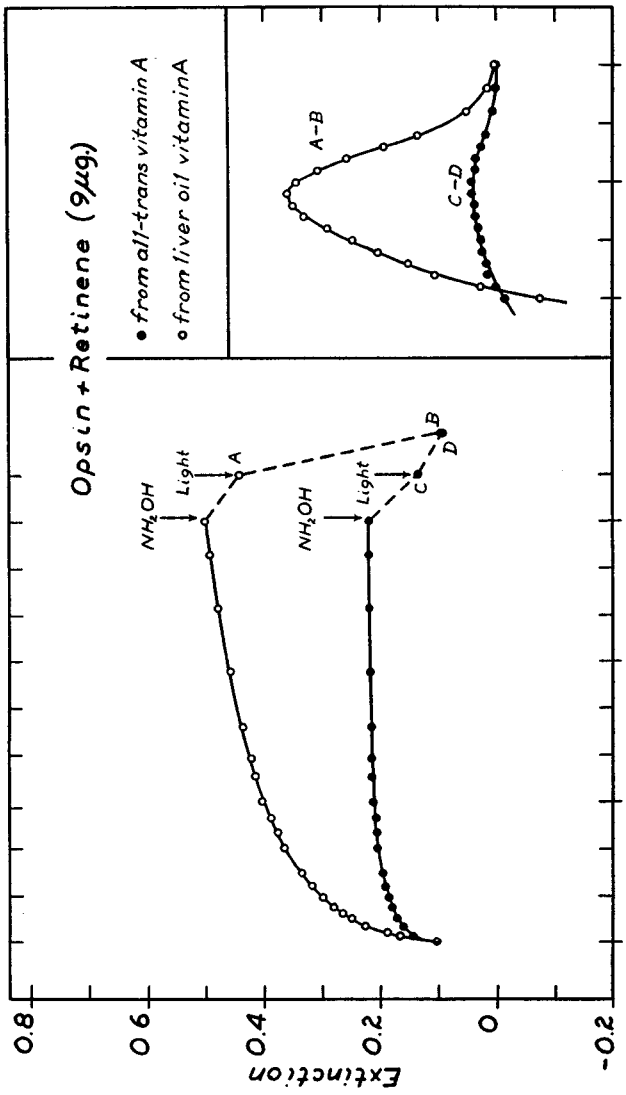

FIG. 6. The isomer specificity of opsin. Synthesis of rhodopsin from opsin and retinene, derived either from crystalline all-trans vitamin A or from fish liver oil concentrate. This figure is otherwise comparable with Fig. 3. On the left are shown changes in the extinction at 500 mμ, on the right the difference spectra of the rhodopsin which had formed. The retinene derived from all-trans vitamin A yielded only a trace of rhodopsin, while that obtained from liver oil vitamin A had formed rhodopsin effectively.

genase, about 40 μg. each, were incubated with 155 μg. of reduced cozymase and 13 μg. of retinene, prepared by the oxidation on solid manganese dioxide of either all-trans or liver oil vitamin A (pH 6; 20°C.). The rates of reduction of both types of retinene are of the same order, but the retinene prepared from all-trans vitamin A is reduced a little more rapidly. The difference in this instance is perhaps too small to be significant.

The isomer specificity of alcohol dehydrogenase presents problems to which we shall return at some future time. It is already clear, however, that this enzyme acts efficiently upon both all-trans and liver oil vitamin A. Such small specificity as it displays is to the advantage of the all-trans isomer. It is therefore opposite in direction to the isomer specificity observed in the over-all synthesis of rhodopsin.

This result leaves little choice but to assume that the main site of isomer specificity in rhodopsin synthesis is the condensation of retinene with opsin. Our first attempts to explore this reaction, however, yielded confused results. Different samples of the same preparation of all-trans retinene, for example, varied as much as 100 to 200 per cent in activity.

We finally realized that the source of these variations is that *retinene is isomerized by simple exposure to light.* No iodine or other catalyst is needed for this reaction. In all procedures involving single stereoisomers of retinene, it must be kept shielded from light if isomerization is to be avoided.

On taking this precaution we succeeded in obtaining from all-trans vitamin A a preparation of retinene which was almost entirely inactive in rhodopsin synthesis. The comparison of this material with the retinene derived from liver oil vitamin A is shown in Fig. 6.

Experiment.—Two samples, each of 9 μg. retinene, obtained by oxidizing either all-trans or liver oil vitamin A on manganese dioxide, were mixed with equal amounts of cattle opsin in 0.5 ml. of 1.4 per cent digitonin solution. At all times during this procedure the retinene preparations were kept either in the dark or in red light, which, since it is not absorbed by retinene, has no effect upon it. Both mixtures were incubated in the dark at 22°C., pH 6.9. The changes in extinction at 500 mμ (rhodopsin) are shown at the left of Fig. 6. After 91 minutes in the dark the difference spectra of both solutions were measured. These are shown at the right of the figure.

Fig. 6 closely resembles Fig. 3 above, in which a similar experiment was performed with corresponding preparations of vitamin A. In the former instance about 10 times as much rhodopsin was synthesized from liver oil vitamin A as from the all-trans isomer. In the present experiment about the same proportions of rhodopsin are synthesized from the corresponding retinenes. It can be concluded that the coupling of retinene with opsin accounts adequately for the isomer specificity of rhodopsin synthesis.

Our problem therefore is to identify the stereoisomer of retinene which is the precursor of rhodopsin. The experiment just described suggests that this is not

all-trans retinene. We proceeded therefore to isolate the geometrical isomers of retinene, and to examine the capacity of each of them to synthesize rhodopsin.

Crystalline Geometrical Isomers of Retinene

All-trans retinene was crystallized in our laboratory by Mr. Robert Gregerman, using essentially the procedure of Ball, Goodwin, and Morton (1948). Crystalline all-trans vitamin A was oxidized to retinene on manganese dioxide, purified by adsorption on a column of calcium carbonate, and a concentrated solution of the product in petroleum ether (b.p. 30–60°C.) was crystallized at about −70°C. in a bath of dry ice in ethyl alcohol. After recrystallization, this product, obtained in an over-all yield of about 40 per cent, melted at 61–62°C. (corrected). Its $\lambda_{max.}$ in petroleum ether is at 369 mμ, with E (1 per cent, 1 cm.) about 1720; and in ethanol it is at 383 mμ, with E (1 per cent, 1 cm.) about 1600.[10] The absorption spectrum of all-trans retinene in ethyl alcohol is shown in Fig. 7; its spectrum in aqueous digitonin solution is shown in Fig. 10.

Neoretinene *a* was crystallized by Mr. Gregerman following much the same procedure with a preparation made by oxidizing crystalline neovitamin A*a* on a column of manganese dioxide.[11] We have since received further preparations of neoretinene *a* from Distillation Products Industries. The absorption spectrum of this substance in ethyl alcohol is shown in Fig. 7, and its spectrum in aqueous digitonin in Fig. 11. The $\lambda_{max.}$ in ethyl alcohol is at 377 mμ, with E (1 per cent, 1 cm.) about 1215.

Following the crystallization and trial of all-trans retinene and neoretinene *a*, neither of which is effective in rhodopsin synthesis, we made a direct attempt to isolate the active isomer. This proved to be a second, apparently mono-cis retinene. It was isolated by chromatographic adsorption from an isomerate prepared by irradiating all-trans retinene. We shall for the present call it neoretinene *b*, pending the determination of its configuration. Since from our point of view this is the most important of the stereoisomeric retinenes, we shall describe its preparation and properties in detail.

Retinene Isomerate.—The starting material is a solution of all-trans retinene in ethyl alcohol, isomerized by exposure to the light of a 160 watt microscope lamp, filtered through a Corning 3389 or 3060 glass to absorb ultraviolet light, and a Corning 3966 glass or a layer of water to absorb heat radiation. For this procedure, the retinene concentration should not exceed 1 mg. per ml., since low concentrations favor the iso-

[10] $\lambda_{max.}$ is the wave length of maximum extinction, the position of an absorption maximum. E (1 per cent, 1 cm.) is the extinction of a solution which contains 1 per cent of solute, weight by volume (*i.e.*, 10 mg. per ml.), measured in a layer 1 cm. in depth.

[11] Our naming of the retinene isomers is intentionally arbitrary. We hope that in this small stereoisomeric set the actual structures may soon be sufficiently well established to permit the use of the configurational designations, *3*-cis, *5*-cis, and so on.

merization.[12] The irradiation is continued for about 30 minutes. Following this, all further procedures are carried out in red light, except the chromatographic adsorption, which is performed in very dim white light, just bright enough to make the yellow bands of adsorbed retinene visible.

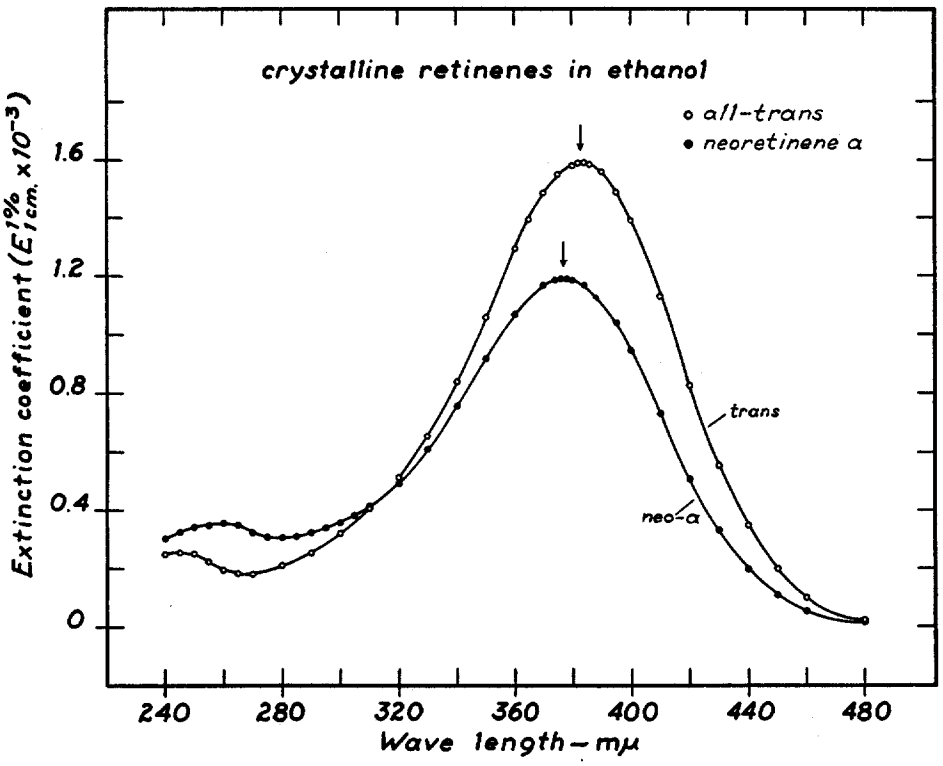

FIG. 7. Absorption spectra of crystalline all-trans retinene and neoretinene *a* in ethyl alcohol. Ordinates show the extinction of a solution containing 1 mg. of retinene per 100 ml., measured in a layer 1 cm. in depth. This is equivalent to the conventional E (1 per cent, 1 cm.) multipled by 10^{-3}.

Chromatography.—Mr. Gregerman made the first attempt to isolate the active isomer. He found the most suitable adsorbent to be acid-washed alumina (*cf.* Wendler, Rosenblum, and Tishler, 1950). Powdered aluminum oxide (Merck reagent, "suitable

[12] In one experiment the proportions of active retinene isomers formed by irradiating all-trans retinene in ethyl alcohol were found to vary with concentration as follows: at 0.1 mg. retinene per ml., about 50 per cent active isomers were formed; at 1 mg. per ml., about 40 per cent; at 10 mg. per ml., about 25 per cent. These solutions were not stirred during irradiation, and the effect may have been caused by the superficial layers of retinene in the more concentrated solutions preventing the isomerizing light from reaching the deeper layers.

for chromatographic adsorption") is washed by stirring into 0.1 N hydrochloric acid. The suspension is filtered under suction and washed on the funnel with distilled water until the washings are no longer acidic. The powder is then dried overnight at about 150°C.

For the adsorption, the powder is poured into a glass tube without tamping, and then is slightly packed by applying suction. The column is wetted with petroleum ether, and the retinene isomerate is poured on in petroleum ether solution. The chromatogram is developed with a mixture of equal parts ethyl ether and petroleum ether. In the course of about an hour a band of adsorbed retinene forms toward the bottom of the column, separated off from a larger, diffuse layer of retinene higher up. This lowermost layer contains the active isomer.

The alumina, prepared as described, contains a little residual water. If it is too dry, the retinene fails to diffuse even in equal parts petroleum ether and ethyl ether; and on such a dry column much of the retinene is destroyed. When the alumina has a slightly higher water content, the chromatogram is developed as described above. With yet more moisture, it can be developed better with mixtures of petroleum ether and benzene than with petroleum ether and ethyl ether. If the alumina is too wet, the retinene moves downward in petroleum ether, making proper development impossible.

Using this procedure, Gregerman obtained preparations of retinene which assayed at 70 to 80 per cent activity. Later, one of us prepared a fraction with activity close to 100 per cent; and from this, by concentration in petroleum ether and cooling to about −70°C., isolated a small amount of crystalline material.

The absorption spectrum of crystalline neoretinene *b* in ethyl alcohol is shown in Fig. 8. The absorption maximum in this solvent is at 377.5 mμ. Not enough material was available to permit an accurate determination of the *E* (1 per cent, 1 cm.) but this appears to lie in the range 900 to 1000.

The most remarkable feature of this spectrum, however, is the second band at 255 mμ. This is what Zechmeister calls a "cis peak," an indication of the presence of a cis linkage. It will be noted in Fig. 7 that all-trans retinene has little absorption in this region, while neoretinene *a* possesses a small cis peak. Neoretinene *b* has by far the highest cis peak of any of the known stereoisomers of retinene.

According to theory, the height of the cis peak is a measure of the degree to which the cis linkage bends the molecule. By this token neoretinene *b* is the most strongly bent of all the configurations of retinene. It may therefore be the *3*-cis isomer.

Other properties of neoretinene *b* are consistent with the same view. In general among carotenoids the geometrical isomer which possesses one centrally located cis linkage has the highest cis peak, the lowest adsorbability, the lowest extinction of the long wave length maximum; and it is formed from and reverts to the all-trans isomer most readily (*cf.* Zechmeister, 1944). Neoretinene *b* has all these properties. Among the known isomers of retinene, it is in a strong position to be assigned the *3*-cis configuration.

With the isolation of this precursor of rhodopsin, our primary task was accomplished. We had now also prepared three of the four geometrical isomers of retinene expected theoretically; only the *3,5*-di-cis isomer was lacking. In

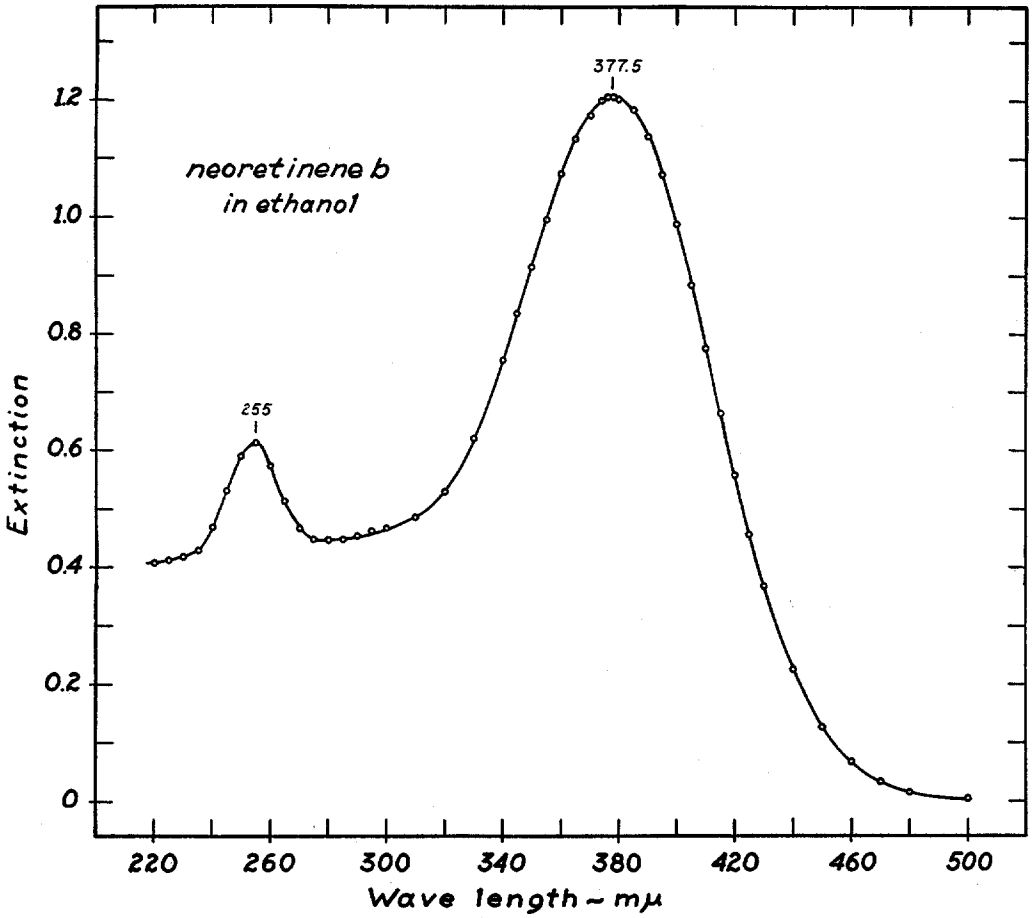

FIG. 8. Absorption spectrum of crystalline neoretinene *b*, the precursor of rhodopsin, in ethyl alcohol. Ordinates show relative values of extinction. This spectrum displays by far the highest cis peak (at 255 mμ) of all the known stereoisomers of retinene.

the meantime, however, the Organic Research Laboratory of Distillation Products Industries had sent us crystalline samples of *two* further isomers—what appeared to be the missing di-cis form, and one other, apparently mono-cis isomer. These substances seem to have a special relation to each other, and for the present we have agreed with the workers at Distillation Products to call them isoretinene *a* and *b*. Their absorption spectra are shown in Fig. 9.

Isoretinene a.—The Distillation Products workers suggest that this is *3*-cis retinene, comparable with that prepared earlier by Graham *et al.* (1949). For this substance they have given us the following data: melting point, 64.5°C.; absorption maximum in ethyl alcohol, 374 mμ; and *E* (1 per cent, 1 cm.) 1270.

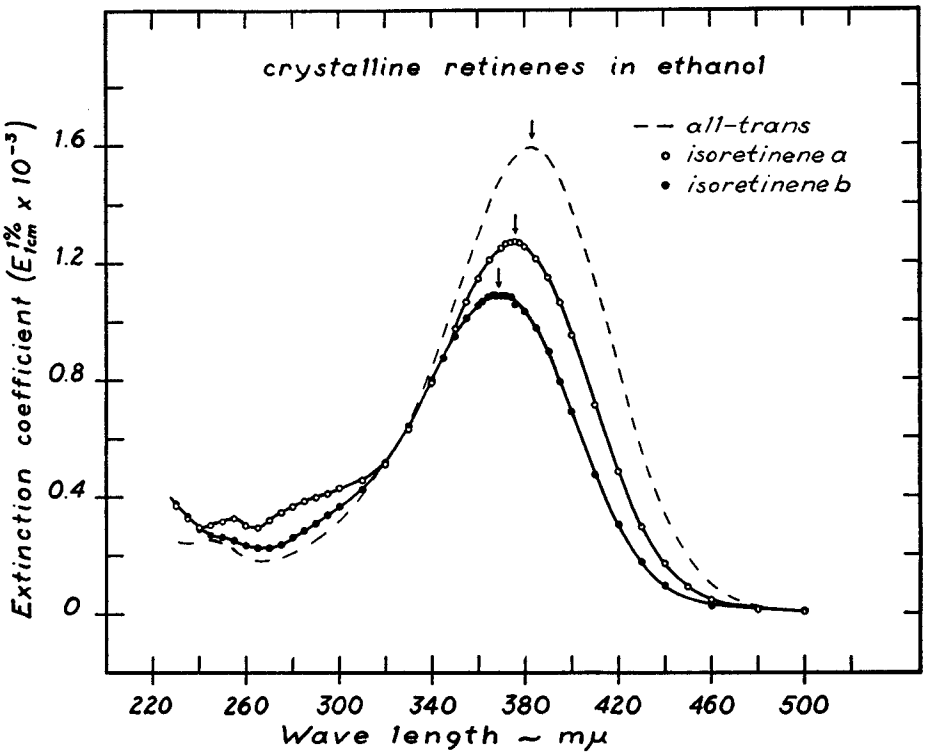

Fig. 9. Absorption spectra of crystalline isoretinenes *a* and *b* in ethyl alcohol, compared with the spectrum of all-trans retinene. The isoretinenes were prepared by the Organic Research Laboratory of Distillation Products Industries. Their displacements of spectrum compared with the all-trans isomer are consistent with the view that isoretinene *a* is a mono-cis, and isoretinene *b* a di-cis retinene.

Isoretinene b.—The Distillation Products workers suggest that this is *3,5*-di-cis retinene, and have given us the following data: absorption maximum in ethyl alcohol, 368 mμ; *E* (1 per cent, 1 cm.) 1090.

These new products bring the total number of apparent stereoisomers of retinene to five, where only four were anticipated. Specifically, we now seem to have three mono-cis retinenes, where only two were expected.

Let us say at once that were it not for the conflict with theoretical expectation, we should have no immediate reason to question the status of any of these substances. As it is, special care must be taken to characterize them. The issue

at stake is that either they are not all geometrical isomers of retinene; or the present theory of cis-trans isomerization in this class of compounds must be expanded.

This is a problem in its own right, and we shall consider it in detail in a subsequent paper (Hubbard, Gregerman, and Wald, 1952–53).

Retinene Stereoisomers in the Synthesis of Rhodopsin

By incubation with opsin, we have tested the capacity of each of the retinene isomers described in the preceding section to synthesize rhodopsin. The methods employed are described in the Appendix.

All-trans Retinene.—Fig. 10 shows the results of an experiment performed with this isomer. A solution of the crystalline substance, incubated in the dark with opsin, yielded very little rhodopsin. This experiment was performed before we had learned to exclude light completely while handling single isomers of retinene; when more adequate precautions are taken, all-trans retinene may yield no rhodopsin at all (*cf.* Fig. 16). A second sample of all-trans retinene, isomerized by 3 minutes' exposure to light, formed rhodopsin efficiently; its activity on assay with excess opsin was about 35 per cent (see Appendix).

The effect of light upon all-trans retinene itself is shown at the right of Fig. 10. After 15 minutes' irradiation, the maximum extinction had fallen 22 per cent, in part because of destruction of the pigment. The absorption maximum had also shifted 5 mμ toward shorter wave lengths. This behavior is specific of the all-trans isomer, and indeed is used below to identify it.

Experiment.—A solution of crystalline all-trans retinene in 2 per cent digitonin was divided into two portions, each containing 9 μg. retinene in 0.18 ml. of solution. One portion was exposed for 3 minutes to the concentrated white light of a 160 watt projection lamp, passing through Corning filters 3389 and 3962 to remove ultraviolet and heat radiation. Both solutions were then incubated in the dark for 90 minutes with 0.32 ml. of cattle opsin solution (pH 6.9; 22–23°C.). Hydroxylamine was added to each mixture in the dark, and the difference spectra were measured. These are shown at the left of Fig. 10. The irradiated retinene had yielded about 7 times as much rhodopsin as the portion which had been protected from light.

The right half of Fig. 10 shows the effect of light upon all-trans retinene dissolved in 1 per cent digitonin. The upper curve shows its absorption spectrum before irradiation, the lower after 15 minutes' exposure to the light described above. The $\lambda_{max.}$ had shifted from 389 to 384 mμ, and had fallen 22 per cent in height. Most of these changes occurred within the first 3 minutes of irradiation, and they were virtually complete within 7 minutes.

Neoretinene a.—Fig. 11 shows the result of a similar experiment with neoretinene *a*. The crystalline substance, incubated with opsin, yielded no rhodopsin. After isomerization by light, its activity was about 35 per cent. As shown at the right of Fig. 11, the absorption spectrum of neoretinene *a* falls in height during irradiation, though less than does that of the all-trans isomer; but the

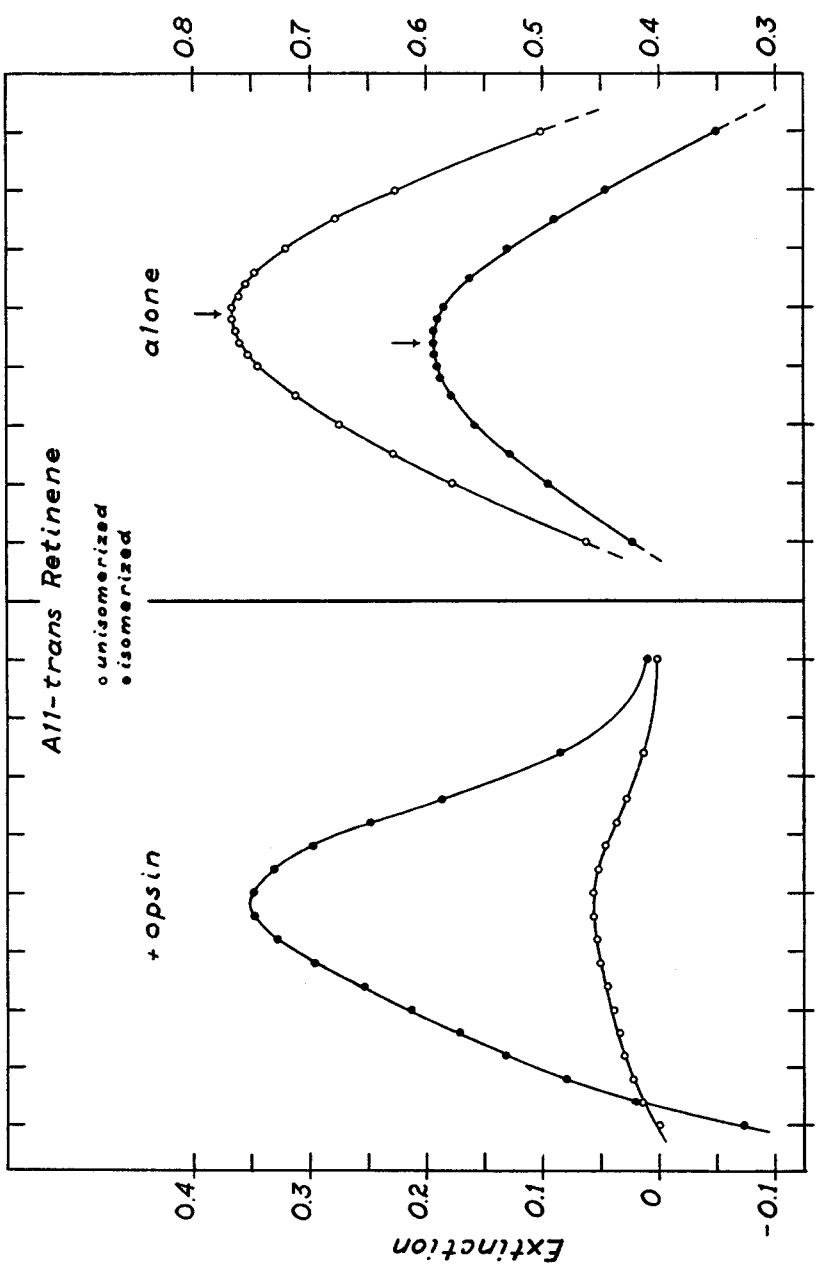

FIG. 10. The effect of isomerization by light upon crystalline all-trans retinene and on its capacity to form rhodopsin. The absorption spectrum of all-trans retinene in aqueous digitonin solution is shown in the upper curve at the right. After isomerization of this substance by 15 minutes' exposure to white light, its spectrum had fallen in height and shifted about 5 mμ toward shorter wave lengths (lower curve). The difference spectra of rhodopsin formed from all-trans retinene and from its isomerate are shown at the left. All-trans retinene yielded only a trace of rhodopsin; after isomerization by light it formed rhodopsin efficiently.

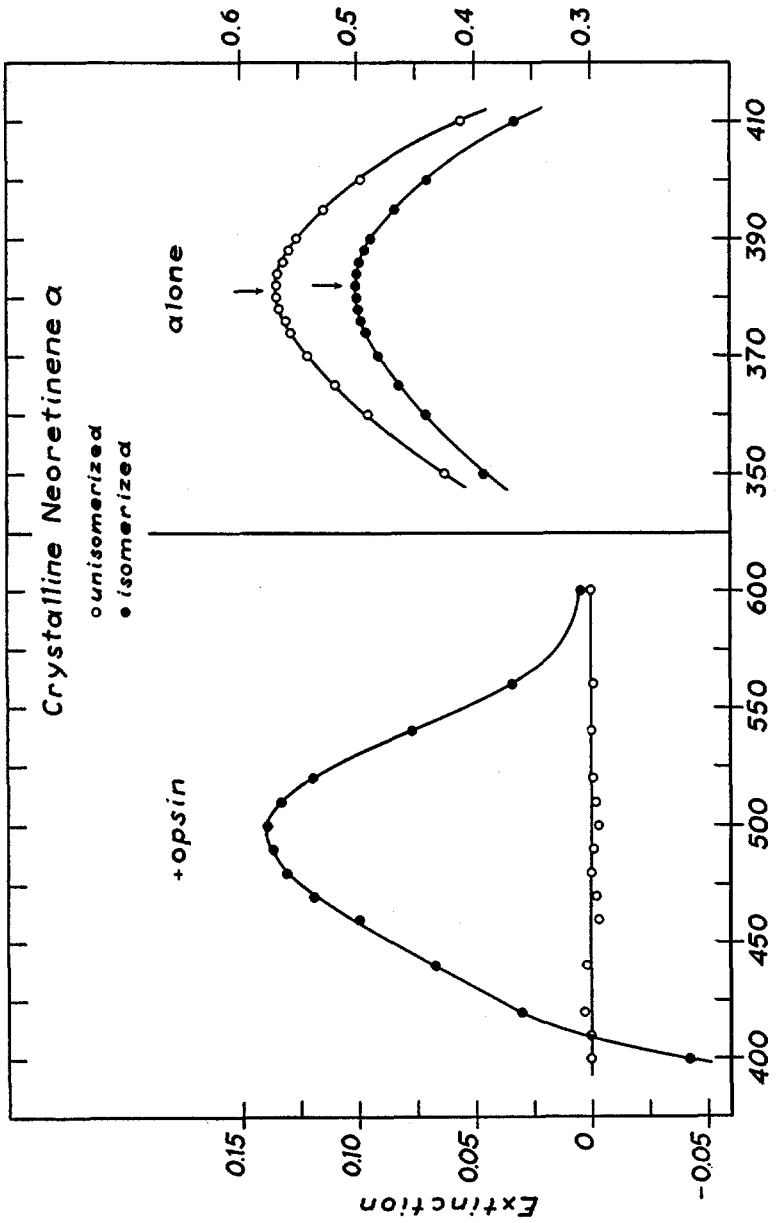

FIG. 11. The effect of isomerization by light upon neoretinene *a* and its activity. The spectrum of crystalline neoretinene *a* in digitonin solution is shown as the upper curve at the right. After isomerization by 15 minutes' exposure to white light, its spectrum had fallen in height and shifted about 1 mμ toward longer wave lengths (lower curve). The difference spectra of rhodopsin formed from neoretinene *a* and from its isomerate are shown at the left. Neoretinene *a* yielded no rhodopsin; but after isomerization by light it formed rhodopsin efficiently.

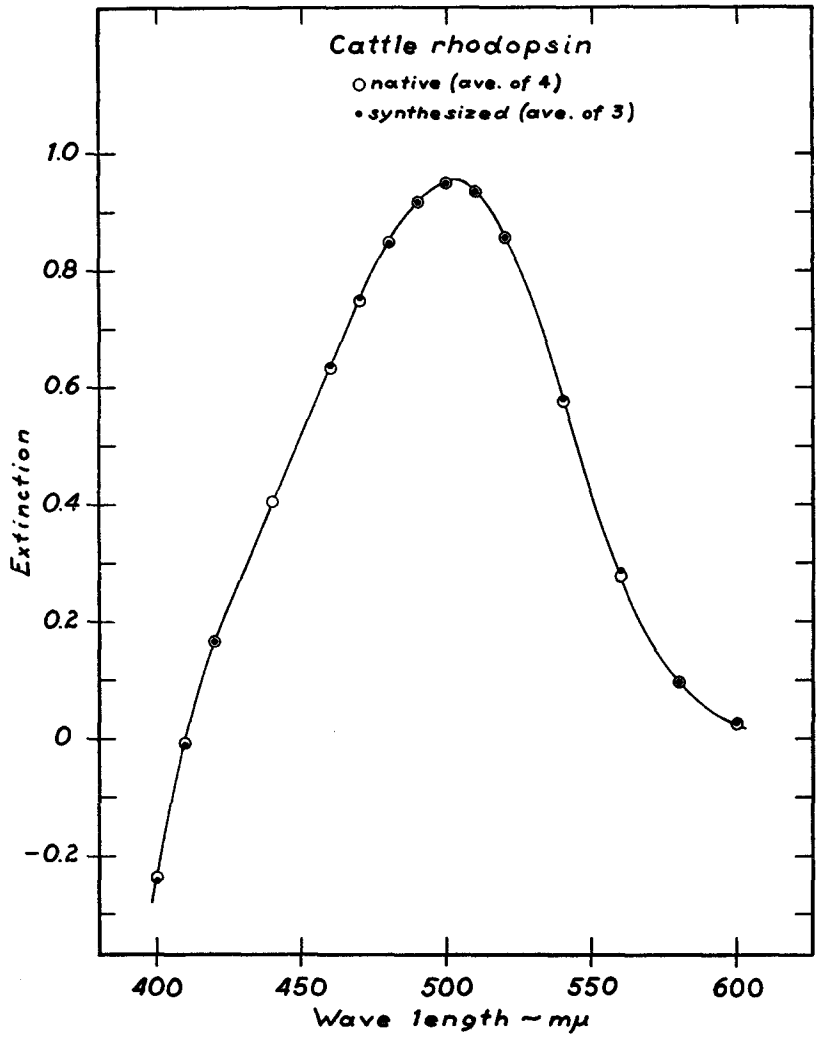

FIG. 12. Difference spectra of cattle rhodopsin extracted from suspensions of dark-adapted rods (average of four experiments), and of rhodopsin synthesized by incubating cattle opsin with neoretinene *b* (average of three experiments). In the latter syntheses, three different preparations of neoretinene *b* and two different opsin preparations were employed. It is plain that the natural and synthetic pigments are identical.

position of the maximum shifts only about 1 mμ toward *longer* wave lengths, arriving close to the position of the isomerate of all-trans retinene.

Experiment.—A solution of crystalline neoretinene *a* in 1 per cent digitonin was divided into two portions, each containing 2.4 μg. in 0.43 ml. of solution. One portion was isomerized by 18 minutes' exposure to the same light as used in the preceding

experiment. Both samples were then mixed with cattle opsin in 1 per cent digitonin to make a final volume of 0.5 ml., and were incubated in the dark for 2 hours (22°C., pH 6.4). Then hydroxylamine was added to each solution, and the difference spectra were measured. These are shown at the left of Fig. 11.

The effect of light upon the absorption spectrum of neoretinene *a* is shown at the right of the figure. The upper spectrum is that of the crystalline isomer in 1 per cent digitonin. After 15 minutes' irradiation as just described, the spectrum had fallen to the lower curve. The maximum extinction had decreased only 12 per cent, compared

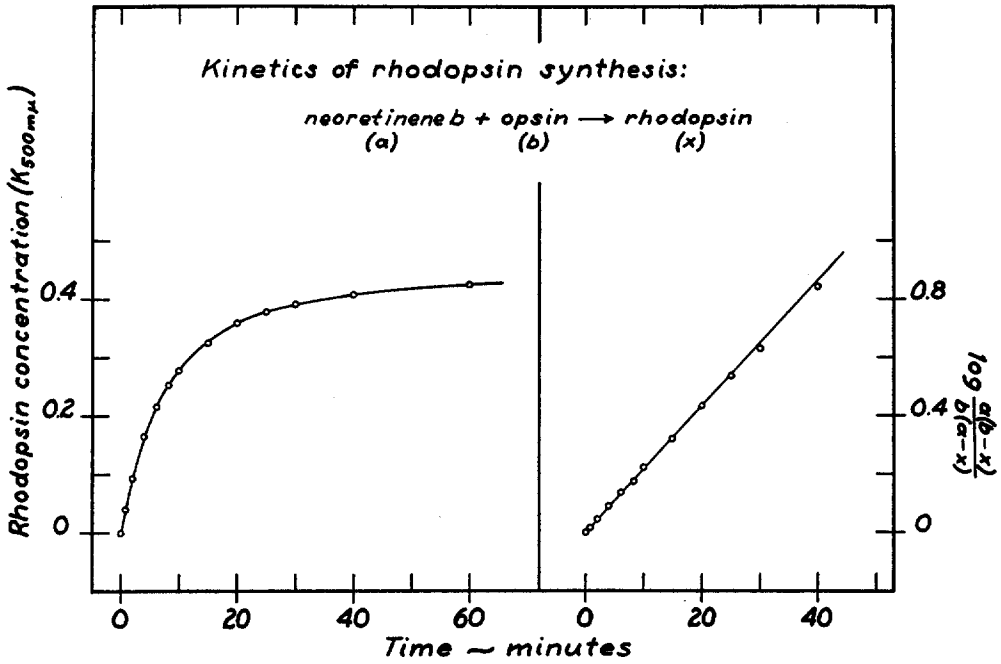

FIG. 13. The synthesis of rhodopsin in solution from neoretinene *b* and cattle opsin. At the left is shown the rise in the extinction at 500 mμ which measures the rhodopsin concentration. The straight line at the right shows that this reaction follows a bimolecular course. In this experiment opsin was slightly in excess (23°C., pH 6.4).

with the 22 per cent change exhibited by all-trans retinene under the same conditions (Fig. 10); while $\lambda_{max.}$ had shifted from 381 to 382 mμ.

Neoretinene b.—On incubation with opsin, this isomer yields a light-sensitive pigment indistinguishable from native rhodopsin. Its difference spectrum is shown in Fig. 12, compared with the spectrum of rhodopsin extracted from dark-adapted rods; and again in Fig. 16. These are the first syntheses of rhodopsin in solution which display the correct absorption spectrum. All previous syntheses had yielded spectra displaced somewhat toward lower wave lengths ("isorhodopsin;" see the next section). Neoretinene *b* is the precursor of the visual pigment.

Kinetics.—The synthesis of rhodopsin from neoretinene *b* and opsin follows the course of a bimolecular reaction. This is shown in Fig. 13. In this experiment the retinene isomer was incubated with about 1.5 equivalents of opsin. The rise of rhodopsin concentration, measured by the extinction at 500 mμ, is shown at the left of the figure. The straight line at the right shows that these data fit the equation for a bimolecular reaction; and is consistent therefore with the view that the synthesis involves the collision of one molecule of retinene with one of opsin.[13]

Experiment.—Neoretinene *b* and cattle opsin were mixed in 1.4 per cent digitonin solution at pH 6.4. The initial extinction of retinene at 385 mμ was 0.436; the initial concentration of opsin could potentially have yielded rhodopsin of extinction 0.695 at 500 mμ. The opsin therefore was about 1.5 times in excess. This mixture was incubated at about 23°C. in the Beckman spectrophotometer. The initial extinction of the mixture at 500 mμ was obtained by adding together the extinctions of opsin and retinene at this wave length. This value, 0.071, was subtracted from all readings.

The data were calculated for the reaction, neoretinene *b* + opsin → rhodopsin, on the basis of the expression:

$$kt = \frac{2.303}{(a - b)} \log_{10} \frac{b(a - x)}{a(b - x)}$$

in which k is the reaction velocity constant, t is time in minutes, a and b are the initial concentrations of neoretinene *b* and opsin; and x is the concentration of rhodopsin. In this calculation, all concentrations are represented by extinctions, to which they are proportional—neoretinene *b* and rhodopsin by their maximal extinctions, and opsin by the maximal extinction of rhodopsin to which it could have given rise.

In the above expression, the first term on the right is a constant, so that the second term, plotted against time, should yield a straight line of slope k. The fact that our observations, plotted in this way, yield a straight line shows this to be a second order reaction.

Isorhodopsin

Isoretinene a.—The incubation of isoretinene *a* with opsin yields a curious result. A light-sensitive pigment is formed, about as quickly and with about the same extinction as if it were rhodopsin; but its absorption spectrum is dis-

[13] Chase and Smith (1939–40) found that the very small regeneration of frog rhodopsin from the products of its bleaching in solution follows the kinetics of a monomolecular reaction. Under the conditions of their experiments, opsin was present in great excess. The synthesis of rhodopsin should therefore have been limited only by the low concentration of active stereoisomers of retinene which was available. This would of course result in first order kinetics in spite of the reaction being intrinsically bimolecular. There is no discrepancy therefore between these observations and our own.

placed 13 mμ below that of rhodopsin, the $\lambda_{max.}$ lying at about 487 mμ (Figs. 14 and 16).

Since the first measurements of the regeneration of rhodopsin in solution by Chase and Smith (1939–40; *cf.* their Fig. 4), it has been apparent that the pigment regenerated under these conditions has its $\lambda_{max.}$ displaced toward shorter wave lengths than rhodopsin extracted from the retina. This difference has appeared in most syntheses of rhodopsin *in vitro*. One sees it clearly, for example, in earlier figures of the present paper.

Collins and Morton (1950), noting this phenomenon in a case of regeneration from the products of irradiating rhodopsin in the cold, suggested that the pigment of displaced spectrum be called *isorhodopsin*. The shift of spectrum ordinarily observed under such conditions is about 5 to 7 mμ. We have indicated elsewhere that this product is a mixture of native with "slightly altered" rhodopsin (Wald, 1951). From this viewpoint the $\lambda_{max.}$ of the "altered" form must be further displaced from rhodopsin than that of the mixture.

It seems appropriate now to attach the term isorhodopsin to the specific light-sensitive pigment formed by the coupling of opsin with isoretinene *a*. This substance may not account for all cases in which rhodopsin is observed to have a displaced spectrum. The term as we propose to use it, however, has the virtue of designating a single molecular species, and aptly characterizes a rhodopsin the prosthetic group of which is isomeric with that of the native pigment.

The synthesis of isorhodopsin from its precursors is shown in Fig. 14. The preparation of cattle opsin used in this experiment had no specific absorption but that of the protein band at 280 mμ. It was mixed with isoretinene *a* in digitonin solution. The spectra shown in the figure represent the opsin and isoretinene *a* in the initial concentrations present in the reaction mixture. The opsin, assayed in a preliminary experiment, was slightly in excess. This solution was incubated in the dark for 1.25 hours at room temperature, pH 6.7. The absorption spectrum of the final product shows that the isoretinene *a* had been completely utilized; only isorhodopsin is left together with a little residual opsin.

The absorption spectrum of isorhodopsin is similar in shape to that of rhodopsin. In addition to the α-band at 487 mμ, and the γ-band at 280 mμ due to the protein, there is a small β-band, as in rhodopsin and porphyropsin, at about 345 mμ.

We have studied also the kinetics of the synthesis of isorhodopsin in solution from opsin and isoretinene *a*. This follows, as in the case of rhodopsin, the course of a bimolecular process. The data look much like those of Fig. 13.

Isoretinene b.—On incubation in the dark with opsin, this presumptive di-cis isomer of retinene seems at first to form no light-sensitive pigment. As the incubation is prolonged over several hours, however, a light-sensitive pigment slowly forms; and this is isorhodopsin. On the other hand, if a solution of iso-

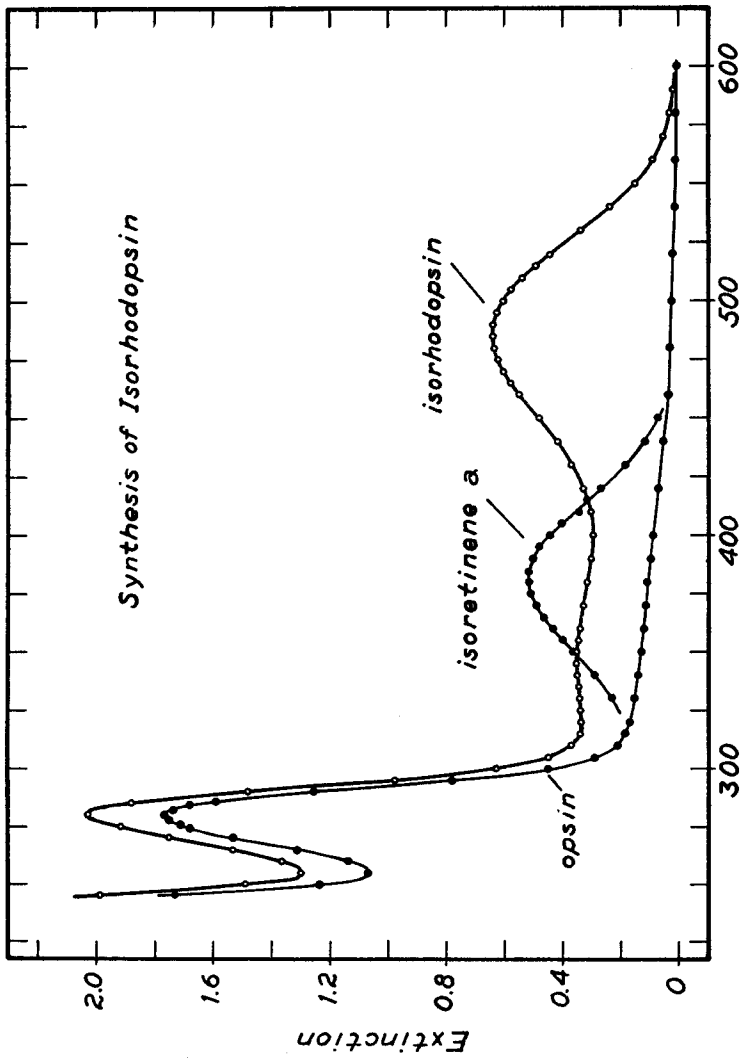

FIG. 14. The synthesis of isorhodopsin from isoretinene *a* and cattle opsin. The absorption spectra of isoretinene *a* and opsin represent the concentrations present initially in the reaction mixture. The spectrum of the final product is that of isorhodopsin mixed with a little residual opsin.

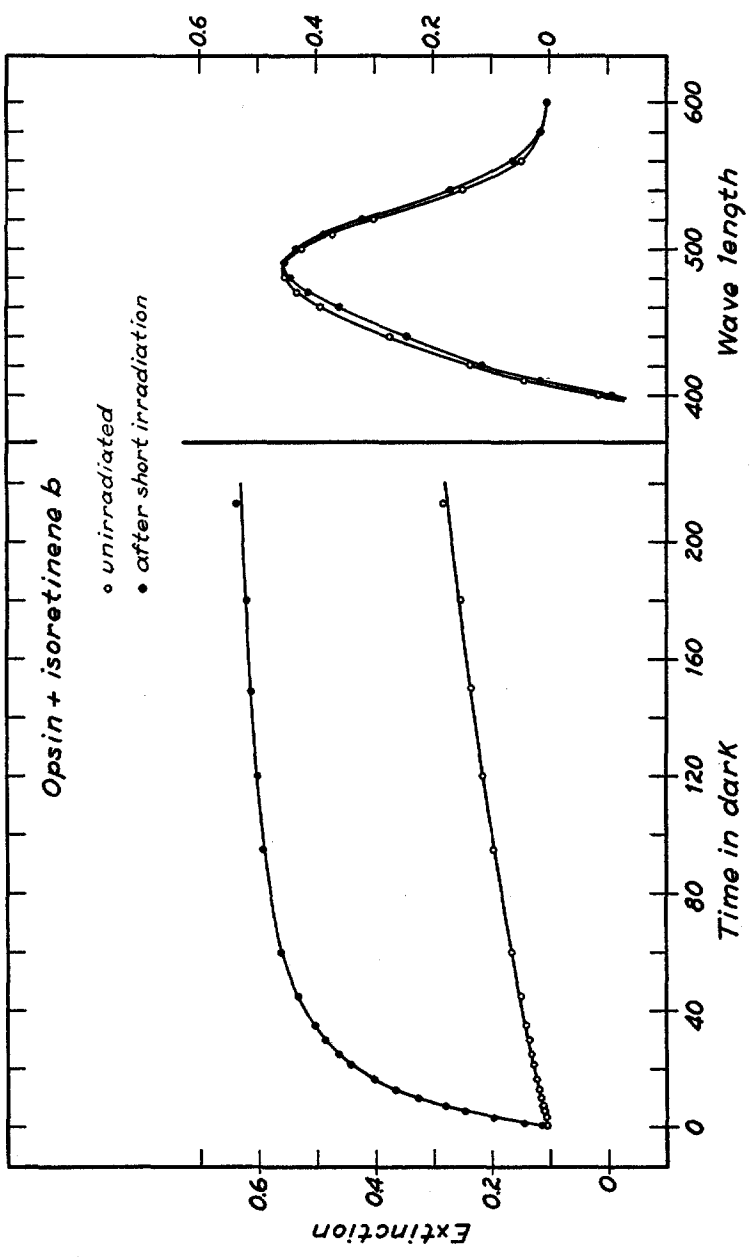

FIG. 15. The behavior of isoretinene *b*. The curves at the left show changes in the extinction at 500 mμ, those at the right the difference spectra of the final products. On incubation with cattle opsin in the dark, isoretinene *b* very slowly forms isorhodopsin (open circles). Following a short exposure to light, isoretinene *b* forms isorhodopsin rapidly (solid circles). This behavior is taken to mean that isoretinene *b* isomerizes preferentially to isoretinene *a*. In the dark this happens slowly, the opsin "trapping" the isoretinene *a* to form isorhodopsin before the isomerization can go further. In the light, isomerization yields isoretinene *a* as a first product, which reacts with opsin to form isorhodopsin.

retinene *b* is exposed to light for 1 to 2 minutes, on incubation with opsin in the dark it yields isorhodopsin rapidly.

Fig. 15 shows such an experiment. On the left is shows the slow "spontaneous" synthesis of isorhodopsin from isoretinene *b*, and the rapid synthesis after this isomer had had a short irradiation. On the right are shown the difference spectra of the products. Isoretinene *b* had formed isorhodopsin with $\lambda_{max.}$ at 486 mμ. After irradiation it had formed predominantly the same pigment; the displacement of $\lambda_{max.}$ to 489 mμ in this instance indicates that the irradiation had produced also a little neoretinene *b*, which formed a trace of rhodopsin.

We conclude from these experiments that isoretinene *b* isomerizes rapidly and preferentially in the light to isoretinene *a*. Longer irradiation is needed to form the other isomers (*cf.* Hubbard, Gregerman, and Wald, 1952–53). Such preferential isomerization occurs also in the dark at room temperature, though much more slowly; and here in the presence of opsin, the isoretinene *a* is "trapped" to form isorhodopsin before the isomerization can go further. Indeed the trapping reaction speeds the isomerization of isoretinene *b* to *a* in the dark; for this occurs more rapidly when opsin is present than in its absence.

Experiment.—A sample of crystalline isoretinene *b* was mixed with excess opsin in the dark, in 1.3 per cent digitonin. Its extinction in 0.5 ml. of the final mixture was 0.41 at 376 mμ. This mixture was incubated in the dark at 22°C. (pH 6.4), and its extinction at 500 mμ was measured periodically to follow the rise of isorhodopsin (Fig. 15, left; open circles). After 530 minutes of incubation, hydroxylamine was added, and the difference spectrum of the product measured. This is shown at the right.

A second sample of isoretinene *b*—twice the above quantity—was irradiated for 2 minutes in the manner described earlier, and was incubated as before with the same amount of opsin. The synthesis of photopigment is shown with solid circles at the left of Fig. 15. After adding hydroxylamine, the difference spectrum at the right was measured. Again it is essentially that of isorhodopsin, though slightly displaced toward longer wave lengths, probably because of a small admixture of rhodopsin.

Recapitulation

The parts which single geometrical isomers of retinene play in the synthesis of rhodopsin and isorhodopsin are brought together in the experiment shown in Fig. 16. Under carefully controlled conditions, all-trans retinene and neoretinene *a* form no light-sensitive pigment. Neoretinene *b* yields rhodopsin; isoretinene *a*, isorhodopsin. It need only be added that as just shown, isoretinene *b* itself is inactive but isomerizes readily to isoretinene *a*, which forms isorhodopsin.[14]

[14] In view of their close similarity of structure, it might be wondered whether inactive geometrical isomers of retinene inhibit competitively the reaction of the active isomers with opsin. This was tested by incubating with opsin a sample of isomerized retinene which was about 40 per cent active, alone and in the presence of

Experiment.—Cattle opsin in digitonin solution was incubated in the dark at room temperature for 2 hours with all-trans retinene (maximal extinction, 0.614) and with a similar solution of neoretinene *a* (extinction, 0.52). In both cases the opsin was present in excess. The final pH was 6.4. After incubation,

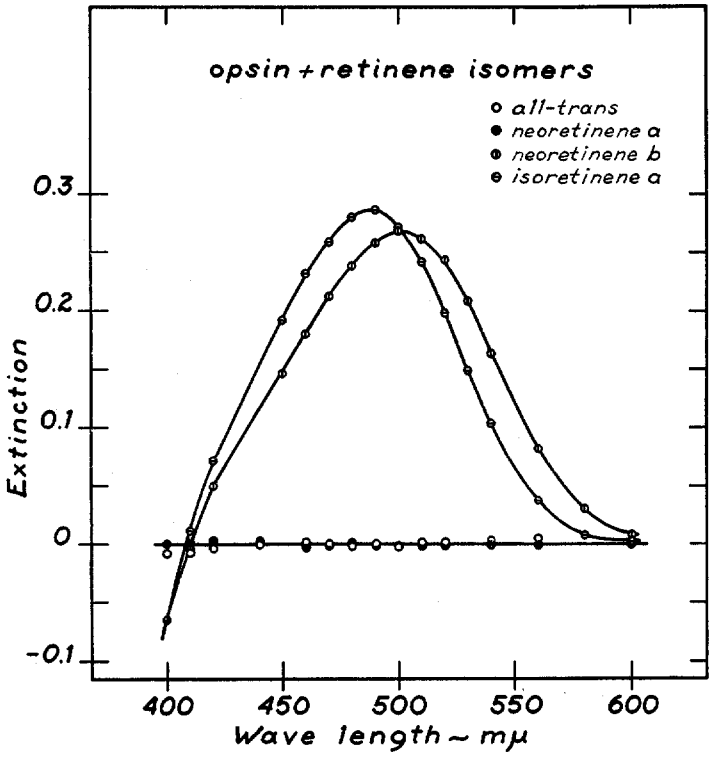

FIG. 16. The reaction of geometrical isomers of retinene with cattle opsin. Difference spectra of the products formed on incubation in the dark with cattle opsin. All-trans retinene and neoretinene *a* yield no light-sensitive pigment; neoretinene *b* yields rhodopsin, and isoretinene *a* isorhodopsin.

hydroxylamine was added to both solutions, and their difference spectra were measured. As Fig. 16 shows, no light-sensitive pigment had been formed.

about 10 times as much all-trans retinene. The all-trans retinene, incubated separately with opsin, yielded an extinction of mixed rhodopsin and isorhodopsin of 0.043; the active isomerate alone, 0.366; and the two together, 0.410; *i.e.*, simply the sum of the separate activities. The all-trans retinene therefore had not interfered at all with the activity of neoretinene *b* and isoretinene *a* present in much smaller concentration in the mixture. Apparently its difference in shape is sufficient to exclude it from attachment to opsin at the site of reaction.

A solution of neoretinene *b* (maximal extinction 0.56) and one of isoretinene *a* (extinction 0.48) were similarly incubated with cattle opsin in the dark for 2¾ hours In. both cases the retinene was present in excess; the syntheses were limited by the opsin concentration. The difference spectra were measured as before. As shown in Fig. 16, neoretinene *b* formed rhodopsin, with $\lambda_{max.}$ 500 mμ, while isoretinene *a* formed isorhodopsin, with $\lambda_{max.}$ 487 mμ. The small difference in extinction is characteristic; isorhodopsin has a slightly higher extinction coefficient than rhodopsin.

The Product of Bleaching

It has been known since Kühne that when the products of bleaching rhodopsin in solution are replaced in the dark, only a small fraction of the pigment is reconstituted. It would seem from this observation alone that retinene may emerge from the bleaching of rhodopsin in another configuration from that which enters its synthesis.

As a first approach to this problem we tested the activity of retinene obtained by bleaching rhodopsin *chemically* in the dark. A concentrated solution of rhodopsin was denatured with methyl alcohol, and the retinene liberated was assayed by incubation with excess opsin. It formed only about one-fifth as much rhodopsin as should have been regenerated from active isomers of retinene.

This problem can be approached more simply however. Retinene is isomerized only by light that it can absorb. Yellow or orange light is absorbed by rhodopsin, and hence bleaches it; such light is not absorbed by retinene, and hence does not isomerize it. If rhodopsin were bleached with such a non-isomerizing light, the retinene which emerged would remain unaltered.

Fig. 17 shows such an experiment. The difference spectrum of a solution of cattle rhodopsin is shown in curve *a*. On bleaching with orange light, its difference spectrum fell to *b*. After 2 hours of incubation in the dark, this spectrum had not changed (*c*); the products of bleaching had regenerated no rhodopsin. This was the fault of the retinene, not of the opsin; for when a portion of the bleached product was incubated with an active preparation of retinene, it formed a large amount of rhodopsin (*d*). It is clear from this experiment that the bleaching of rhodopsin with a non-isomerizing light yields an inactive configuration of retinene.

Experiment.—A concentrated solution of cattle rhodopsin (extinction 4.0 at 500 mμ) was used to prepare the following mixtures. One portion (0.12 ml.) was diluted to 0.5 ml. with digitonin solution, and 0.1 ml. of 1 M hydroxylamine added in the dark (*a*). The remaining rhodopsin was bleached 40 seconds with the bright orange light of a 160 watt projection lamp filtered through Corning glasses 3482 and 3966. All further procedures were carried out in dim red light.

The bleached solution was divided into three equal parts, each of 0.12 ml. One was mixed quickly with digitonin and hydroxylamine in the same proportions as (*a*);

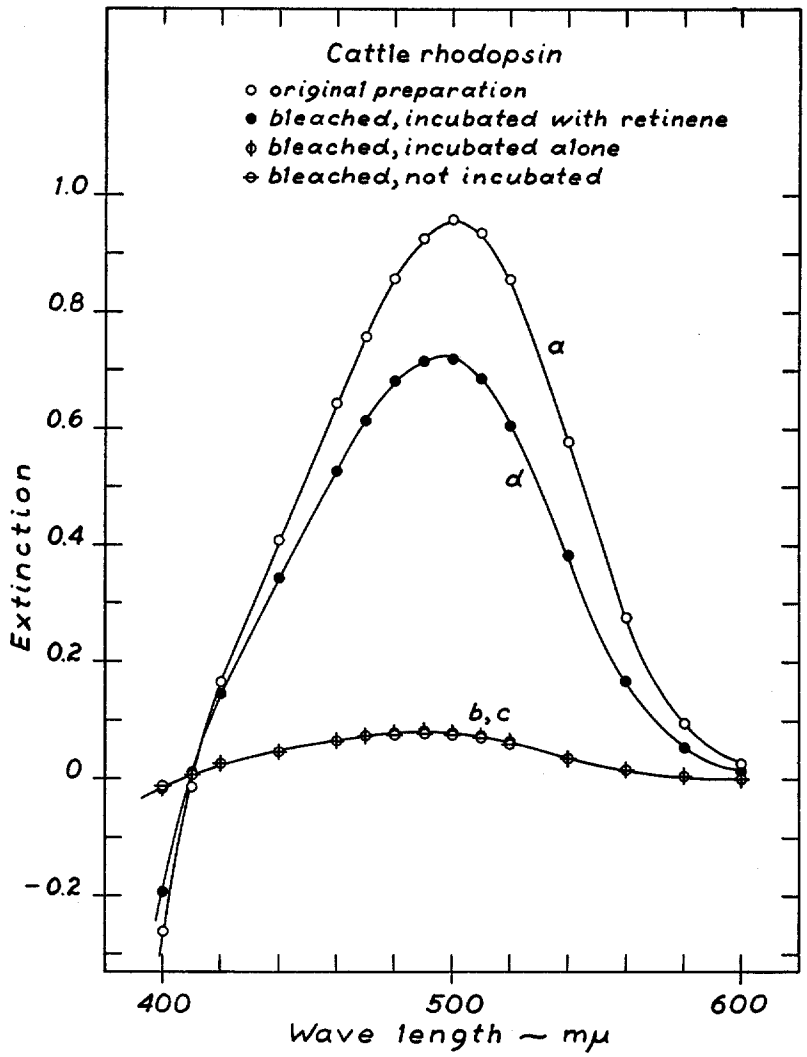

FIG. 17. The bleaching of rhodopsin in orange (*i.e.*, non-isomerizing) light yields an inactive isomer of retinene. The difference spectrum of the original rhodopsin is shown in (*a*). On bleaching with orange light the difference spectrum fell to (*b*). After 2 hours of incubation in the dark, this spectrum had not changed (*c*); the products of bleaching in orange light had regenerated no rhodopsin. This was the fault of the retinene, not of the opsin, for on adding an active retinene preparation to the bleached product, a considerable amount of rhodopsin was synthesized (*d*).

mixing was completed within 45 seconds after bleaching (sample *b*). A second portion was diluted to 0.5 ml. with digitonin and incubated in the dark for 2 hours at room temperature, pH 6.4 (sample *c*). The third portion was diluted to 0.5 ml. with a digi-

tonin solution of isomerized retinene, and was similarly incubated for 2 hours (sample *d*). After incubation, hydroxylamine was added to samples *c* and *d*, and the difference spectra of all four samples were measured. These are shown in Fig. 17.

This experiment clarifies a peculiar observation reported some years ago by Chase (1937) and Chase and Smith (1939–40). These workers found that solutions of rhodopsin bleached with light containing blue and violet components of the spectrum regenerated small amounts of rhodopsin on incubation in the dark; but rhodopsin bleached in yellow light did not regenerate. They reported also that when rhodopsin is bleached in yellow light, subsequent exposure of the yellow product to blue light causes it to bleach a little further. They concluded that their preparations contained a yellow photosensitive pigment, the decomposition of which by light aided the regeneration of rhodopsin.

It is now clear that this pigment is retinene, and that the action of light is not to decompose but to isomerize it. Since the bleaching of rhodopsin yields inactive retinene, this must be isomerized before regeneration can occur. Both blue and yellow lights can bleach rhodopsin, but only the blue can go on to isomerize retinene.

These relations are demonstrated in Fig. 18, which repeats under more favorable conditions an experiment first performed by Chase and Smith. A rhodopsin solution was divided into two parts. One was bleached in orange light, the other in white light, and both were put back in the dark to regenerate. The difference spectra of the products are shown at the left. The solution bleached in orange light regenerated 7 per cent, the other 26 per cent. Now the regenerated rhodopsin was bleached again, but with the lights reversed: the solution first bleached in orange light was rebleached in white light, and *vice versa*. After a second incubation in the dark it was found that the regenerations had also reversed. The sample which had first regenerated 7 per cent now regenerated 26 per cent; that which had first regenerated 26 per cent now regenerated 8 per cent. It is plain that the extent of regeneration depends simply upon the degree to which the light used to bleach rhodopsin can go on to isomerize retinene.

Experiment.—Two samples of a solution of cattle rhodopsin were bleached completely by irradiating for 20 minutes either with white light (400 to 700 mμ) or with orange light (540 to 700 mμ). Both solutions were incubated in the dark without anything being added, for 1.5 hours at 23°C. Then portions of each were removed, hydroxylamine added, and the difference spectra measured. These are shown at the left of Fig. 18.

The remainder of each of these solutions was now rebleached, but with the lights interchanged. Again both solutions were incubated in the dark as before, and their difference spectra measured. These are shown at the right of the figure.

What is the configuration of the inactive retinene liberated in the bleaching of rhodopsin?

This is shown in Fig. 19. It will be recalled that when all-trans retinene is

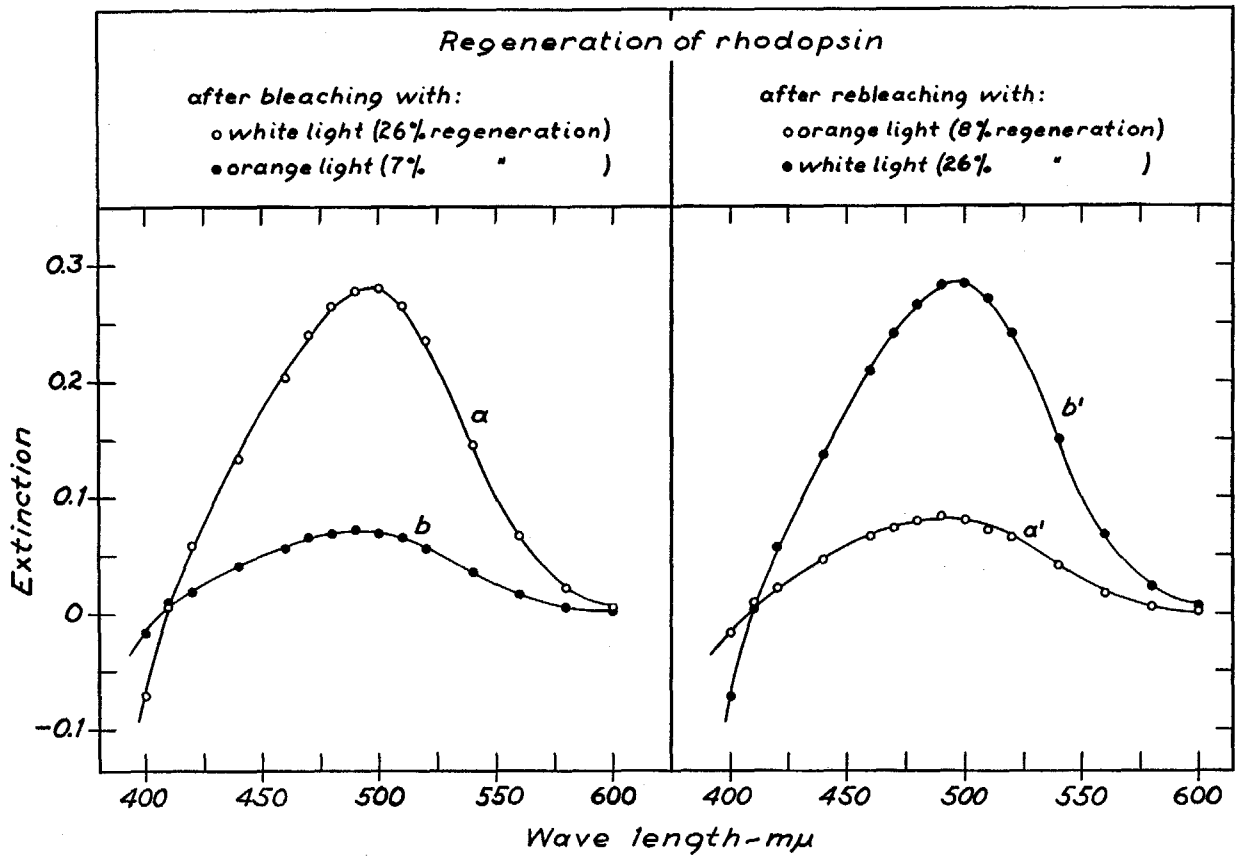

FIG. 18. The regeneration of rhodopsin from the products of bleaching with orange (*i.e.*, non-isomerizing) and with white (*i.e.*, isomerizing) lights. Two samples of a solution of cattle rhodopsin were bleached exhaustively with these lights, and the products were incubated in the dark. The curves at the left show the difference spectra of the regenerated rhodopsin. The regenerated pigments were bleached a second time, but with the lights reversed, and the products incubated in the dark as before. The curves at the right show the rhodopsin regenerated after the second bleaching. In both instances the solutions bleached in orange light had regenerated only 7 to 8 per cent, while those bleached in white light had regenerated 26 per cent. The amount of regeneration depends upon the extent to which the lights used to bleach rhodopsin can go on to isomerize retinene.

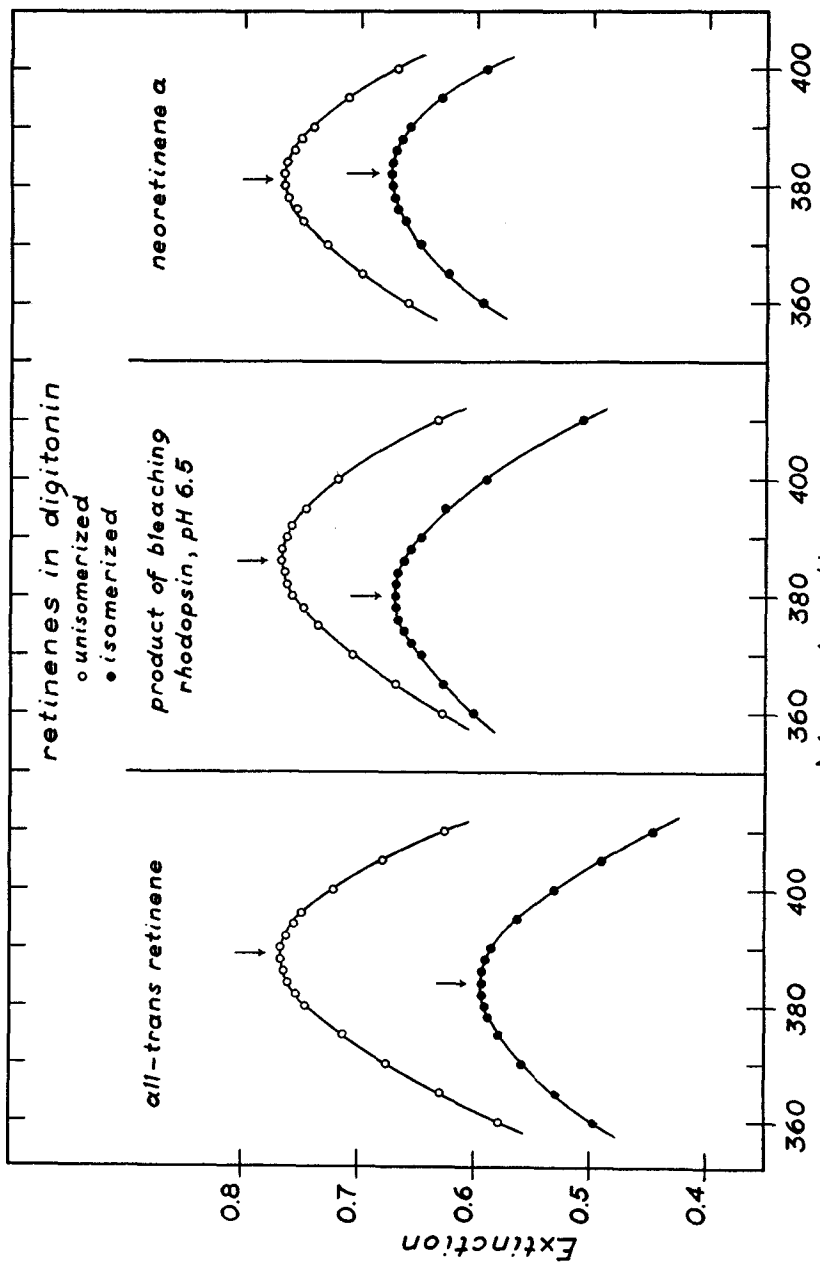

FIG. 19. Configuration of the retinene liberated from rhodopsin by bleaching with a non-isomerizing light. When all-trans retinene is isomerized by light, its $\lambda_{max.}$ shifts about 5 mμ toward shorter wave lengths (left). This is unique behavior. On isomerization, the $\lambda_{max.}$ of neoretinene *a* or *b* or isoretinene *a* shifts about 1 mμ toward *longer* wave lengths (*right*); while that of isoretinene *b* shifts 7 to 9 mμ toward longer wave lengths. As shown in the central panel, when the product of bleaching rhodopsin in orange light (upper curve) is exposed to while light, its $\lambda_{max.}$ falls in extinction and shifts about 5 mμ toward shorter wave lengths (lower curve). This behavior characterizes all-trans retinene.

isomerized by light, its absorption maximum is displaced 5 to 6 mμ toward *shorter* wave lengths (Fig. 11; Fig. 19, left). The isomerization of neoretinene *a* or *b* or isoretinene *a* leaves the position of the absorption maximum almost unchanged (*cf.* Fig. 19, right). The isomerization of isoretinene *b* moves its $\lambda_{max.}$ 7 to 9 mμ toward *longer* wave lengths.

The behavior of the product of bleaching rhodopsin with a non-isomerizing light is shown in the central panel of Fig. 19. On irradiation with an isomerizing light, its spectrum shifts 5 to 6 mμ toward shorter wave lengths. This behavior uniquely characterizes all-trans retinene.

The bleaching of isorhodopsin with a non-isomerizing light yields the same result. On irradiating the bleached product with an isomerizing light, its spectrum shifts about 5 mμ toward shorter wave lengths. This product also appears to be all-trans retinene.

It can be concluded that retinene emerges from the bleaching of rhodopsin or isorhodopsin predominantly or exclusively all-trans. Since retinene enters these pigments in other configurations, the cycle of their synthesis and bleaching has as one consequence the isomerization of retinene.

Experiment.—The left and right hand panels of Fig. 19 are taken from Figs. 10 and 11; the ordinates of all the spectra have been multiplied by suitable factors to bring the absorption maxima of the initial isomers to the same height.

The central panel is based upon the following experiment. To a rhodopsin solution in 1 per cent digitonin (pH 6.5), *p*-chloromercuribenzoate (2.5×10^{-4} M) was added to block regeneration (Wald and Brown, 1951–52). Except for the irradiations described below, all procedures were carried out in dim red light. The rhodopsin was bleached in orange light until further exposure did nothing (5.5 minutes), and then left at room temperature to complete all dark changes. The spectrum of the bleached product, measured in the region of retinene absorption, is shown in the upper curve. After 15 minutes' exposure to the same white light as had been used to irradiate the isomers of the right and left hand panels, its absorption spectrum had changed to the lower curve.

An identical experiment with isorhodopsin, prepared by incubating isoretinene *a* with cattle opsin, yielded essentially the same result.

DISCUSSION

The Rhodopsin Cycle.—Retinene enters rhodopsin in one configuration and emerges in another. It goes in as neoretinene *b;* it comes out primarily or wholly all-trans. Before it can regenerate rhodopsin it must be re-isomerized to the active form. An isomerization cycle is therefore an intrinsic component of the rhodopsin system.

This is shown in Fig. 20. The all-trans retinene which results from bleaching rhodopsin cannot contribute to its regeneration until isomerized in some way to neoretinene *b*. Before this process is completed, much of the retinene is reduced to all-trans vitamin A; and this in turn must be isomerized to neovitamin A*b* before it can reenter rhodopsin synthesis.

Reversibility of Bleaching.—It is fundamentally this relationship that has made it so difficult in the past to reverse the bleaching of rhodopsin *in vitro*. We can see now that what little success was achieved in this process by Kühne and later workers was due to whatever fortuitous isomerization of vitamin A or retinene occurred in the course of their experiments. The same is true of our recent syntheses of rhodopsin in retinal homogenates from products of its bleaching (Wald and Hubbard, 1950; Hubbard and Wald, 1951). They succeeded only to the degree that, by one means or another, some vitamin A or retinene was isomerized from the all-trans to active forms.

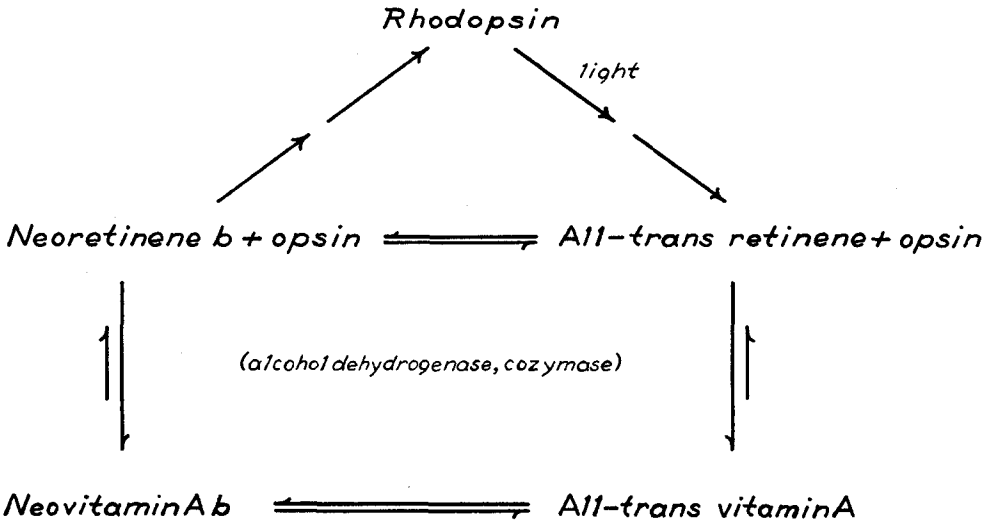

FIG. 20. The rhodopsin cycle. The bleaching of rhodopsin yields all-trans retinene, which must be isomerized to neoretinene *b* before it can regenerate the visual pigment. Alternatively, having been reduced to all-trans vitamin A, the latter in turn must be isomerized to neovitamin A*b* before it can take part again in rhodopsin synthesis. Rod vision depends therefore upon the continuous stereoisomerization of all-trans retinene or vitamin A, or the continuous replacement of these substances by new supplies of neovitamin A*b* from external sources.

This is also the point of our having "flooded" opsin or bleached rhodopsin with added retinene to promote regeneration (Wald and Brown, 1950). A large excess of retinene was needed to make a little rhodopsin, and when the synthesis had come to an end, most of the retinene remained unused. It is clear now that for the most part we had added the wrong configurations of retinene. Only a small fraction was in the form of the active isomers, neoretinene *b* and isoretinene *a*, and from these we obtained mixtures of rhodopsin and isorhodopsin.

When, as in the present experiments, rhodopsin and isorhodopsin are synthesized from the single active isomers of retinene, the reaction is stoichiometric. When an equivalent of opsin is present the retinene is completely utilized, and

none of it remains in the reaction mixture at the end of the synthesis (*cf.* Fig. 14).

A Second Photochemical Process.—The isomerization of retinene by light—specifically by the blue, violet, and ultraviolet light which it can absorb—is a second and heretofore unsuspected photochemical reaction in the rhodopsin system.

When the all-trans retinene which emerges from the bleaching of rhodopsin is isomerized by light, its $\lambda_{max.}$ falls in extinction and shifts 5 to 6 mμ toward shorter wave lengths (Fig. 19, central panel). These changes account for the observation of Chase (1937) and Chase and Smith (1939–40) that after rhodopsin is bleached in yellow (*i.e.*, non-isomerizing) light, the product bleaches a little further on exposure to blue or violet (*i.e.*, isomerizing) light.

Dartnall (1952) has recently reported finding a "visual pigment 467" in extracts of the tench retina. The extracts, which contained primarily porphyropsin, were bleached as far as they would go in red light. The product, on subsequent exposure to white light, bleached slightly further. The difference spectrum of this second change was maximal at 467 mμ, and was taken to indicate the presence of a new visual pigment.

It seems probable that, other considerations apart, the second bleaching involved the isomerization of retinene$_2$, liberated from porphyropsin by the initial exposure to red light. Indeed Dartnall's Fig. 4 shows that the difference spectrum of the retinene$_2$ which first emerged had its $\lambda_{max.}$ at about 405 mμ, and that on exposure to white light this shifted to about 400 mμ (in Dartnall's figure these maxima appear as minima). This is exactly the amount the spectrum is displaced when one isomerizes the all-trans retinene$_1$ which comes out of rhodopsin.

The data shown in the central panel of our Fig. 19 result from the same type of experiment as Dartnall describes, done in this case with rhodopsin and at a more acid pH. If in this figure one subtracts the lower from the upper curve—the spectrum of the isomerized from that of the unisomerized product of bleaching—one obtains a difference spectrum with a sharp peak at 410 mμ, in Dartnall's terminology a "visual pigment 410." This is, however, a meaningless procedure; there lies behind it only the isomerization of retinene.[15]

[15] Something should be said here concerning the general status of difference spectra. They can always be used as arbitrary indications of a change in the direct spectrum; but this change resembles the absorption spectrum of the original material only when the latter changes to something far different. So for example, the difference spectrum of rhodopsin bears some resemblance to its absorption spectrum only because rhodopsin, with $\lambda_{max.}$ at 500 mμ, bleaches to a product with $\lambda_{max.}$ at about 385 mμ. Even in this case there is considerable distortion. The closer together are the initial and final direct spectra from which a difference spectrum is computed, the greater is the distortion. When as in the present instance the direct spectra are only 5 mμ apart, the difference spectrum is all distortion, and has no relation whatever to an absorption spectrum.

The Isomerization Cycle in Vivo.—Light has only a limited opportunity to isomerize retinene in the eye. Much of it is first reduced to vitamin A, which absorbs, and hence might be isomerized only by wave lengths of the ultraviolet too short to penetrate to the retina (Wald, 1952).

For the most part the isomerization cycle is probably completed in other ways. One important factor may be the exchange of stereoisomers of vitamin A between the retina and the blood circulation. Some years ago we obtained direct evidence that vitamin A diffuses into and out of the retina (Wald, 1935–36). Frog retinas light-adapted *in vivo* were found to contain only one-third to one-fifth as much vitamin A as retinas removed from the eye in the dark-adapted condition and then bleached. It was concluded that during light adaptation *in vivo* more vitamin A is liberated than the retina can hold, and that the excess diffuses out into contiguous tissues and the circulation. During dark adaptation the retina recaptures vitamin A by binding it in non-diffusible form in rhodopsin. Independent histological evidence has since been provided for these changes (von Jancsó and von Jancsó, 1936; Greenberg and Popper, 1941; Johnson and Detwiler, 1942).

We can add now that such exchanges are stereochemically selective. During visual activity the retina presumably discards into the circulation primarily the all-trans vitamin A which emerges from the bleaching of rhodopsin, and in forming rhodopsin it binds specifically neovitamin A*b*. These interchanges keep the visual processes intimately connected with the metabolism and transport of vitamin A throughout the organism, with the vitamin A nutrition, and with the animal's total capacity to stereoisomerize this substance.

Mechanism of Isomerization in Vivo.—Whatever its importance in the eye, light can play no large part in the stereoisomerization of vitamin A in the body as a whole. Another process that might be important here, however, is thermal isomerization, particularly in a warm blooded animal. A general procedure for stereoisomerizing carotenoids is to heat them in solution. Some of them stereoisomerize even at room temperature, though in general very slowly. On refluxing solutions of all-trans carotenoids in benzene or petroleum ether (b.p. 60–80°C.) stereoisomeric equilibrium is usually attained within 15 to 60 minutes (Zechmeister, 1944).

If it were true that vitamin A stereoisomerizes at 60–80°C. within an hour, the same change might occur at mammalian body temperature within a day. We find, however, that all-trans retinene isomerizes very slowly on heating, 3 hours at 70°C. having almost no effect upon it (Hubbard, Gregerman, and Wald, 1952–53). If the same is true of vitamin A, thermal isomerization is a very slow process indeed at body temperature.

This consideration lends special point to the possibility that there exists a stereoisomerizing enzyme—a vitamin A or retinene isomerase. Such an enzyme in the eye tissues would greatly simplify the operation of the rhodopsin system. Bliss (1951) and we (Hubbard and Wald, 1951) have reported extracting

from the combined pigment epithelium and choroid layers of the frog eye water-soluble, heat-labile factors, probably proteins, which promote the regeneration of rhodopsin *in vitro* from the products of its bleaching. It is possible that these factors act in part by catalyzing the stereoisomerization of retinene and vitamin A.

Isorhodopsin an Artefact.—Our stereoisomerizations of vitamin A or retinene *in vitro* have always yielded mixtures containing both the neo-*b* and iso-*a* isomers, and from these we have always synthesized mixtures of rhodopsin and isorhodopsin. The retina *in vivo*, however, makes rhodopsin alone. Some process which supplies vitamin A for rhodopsin formation must act selectively to single out the neo-*b* isomer or to exclude isoretinene *a*. It seems important that in our earlier experiments, homogenates of retina, or of retina with pigment layers, working upon *their own content* of vitamin A, tended to make rhodopsin; while on supplementation with liver oil concentrates they made mixtures of rhodopsin and isorhodopsin (Wald and Hubbard, 1950; Hubbard and Wald, 1951). If any considerable proportion of the isomerization of vitamin A or retinene occurs enzymatically, in the eye or elsewhere, it is possible that this process converts the all-trans isomer selectively to the neo-*b* configuration.

In any case it should be recognized that isorhodopsin has as yet no status in the living organism, and must for the present be regarded as an artefact. It is possible that isovitamin A*a* and *b* are in the same position, for neither substance has yet been demonstrated to occur *in vivo*.

The Structure of Rhodopsin.—In considering the nature of the linkage between retinene and opsin in such a molecule as rhodopsin, one tends to concentrate attention upon the reactive aldehyde group of retinene. The decisive importance of the configuration of retinene in forming this molecule, however, shows that its hydrocarbon chain also is involved. This could mean that specific points of attachment exist along the chain, perhaps different points in rhodopsin and isorhodopsin. On the other hand, one cannot as yet afford to be overexplicit in thinking about such matters. The conditions which govern the coupling of small molecules with proteins are just beginning to be understood. It seems from certain recent studies of the relations between enzymes and substrates that multiple points of attachment may well be the rule; but there is evidence also that within narrow limits the entire *gestalt* of the small molecule must fit the topography of the protein, so making possible long sections of attachment by van der Waals and other forces.[16]

It was suggested some time ago that the β-band in the absorption spectrum of rhodopsin, at about 350 mμ, may be a cis peak, an indication that the chromo-

[16] Compare for example the structural aspects of the substrate which govern the activity of proteinases (Bergmann and Fruton, 1941; Neurath, 1952); cholinesterase (Nachmansohn and Wilson, 1951); and the acetylcholine "receptor substance" (Welsh and Taub, 1951). In all these cases there is good evidence that extensive areas of attachment bind the substrate to its protein.

phore of rhodopsin possesses a cis linkage (Wald, 1949, p. 99; Wald, 1951). This view is in good accord with the fact that rhodopsin is made from a cis-retinene. It suggests further, however, that the chromophore of rhodopsin still retains the cis configuration.

The same may be said of isorhodopsin. This pigment displays a β-band at about 348 mμ, which again seems to represent a cis peak (Fig. 14).

Another consideration bears upon the same point. If in the process of combining with opsin neoretinene *b* and isoretinene *a* went over to the all-trans configuration, only one light-sensitive pigment should result. Instead there are two. Apparently therefore the configurational distinctions which divide these isomers of retinene still exist in rhodopsin and isorhodopsin.

This means that the transfer to the all-trans configuration must occur at some stage in the process of bleaching. We have distinguished two intermediates in the bleaching of rhodopsin—lumi-rhodopsin, the immediate product of the light reaction; and meta-rhodopsin, the product of a subsequent "dark" reaction (Wald, Durell, and St. George, 1950). The absorption spectra of both substances, obtained by irradiating rhodopsin in the cold, still display cis peaks (St. George, unpublished observations). It should be noted also that when these intermediates are allowed to react further in the dark, they regenerate about half the original amount of photosensitive pigment. Presumably therefore they are not yet predominantly all-trans.

The light-sensitive pigment regenerated under these conditions appears to be a mixture of rhodopsin and isorhodopsin; indeed it is to this product that Collins and Morton first attached the latter term. This implies that a partial isomerization of lumi- or meta-rhodopsin had occurred, leading to the configurations characteristic of both active retinene isomers. One may suppose that the exhaustive irradiation to which lumi-rhodopsin was exposed in these experiments had isomerized its prosthetic group to a mixture of all-trans and cis isomers, about half composed of active configurations. The latter then regenerated a mixture of rhodopsin and isorhodopsin, while the inactive isomers went on to form retinene and opsin.

The outcome of this discussion is that in rhodopsin and isorhodopsin the prosthetic groups appear to be in cis configuration. In lumi- and meta-rhodopsin, the prosthetic groups are probably still initially cis, but can perhaps be isomerized by light. The isomerization to the all-trans configuration seems otherwise to occur between meta-rhodopsin and retinene. In any case the retinene which emerges from rhodopsin and isorhodopsin, whether by chemical destruction in the dark or by bleaching with a non-isomerizing light, is predominantly the all-trans isomer.

The isomerization of retinene in its passage through rhodopsin has important structural inplications. Some years ago Meunier and Vinet (1947), discussing the blue product which vitamin A yields when mixed with antimony chloride, predicted that whatever stereoisomer of vitamin A entered this reaction, a

single all-trans product would result. They proposed that the reaction involves the formation of a vitamin A ion, whose greatly increased resonance in the ionic state is responsible for the blue color, and also forces the molecule into the all-trans configuration which permits resonance the freest play. In good agreement with this hypothesis, we find that all the geometrical isomers of retinene yield with antimony chloride the identical blue product (Hubbard, Gregerman, and Wald, 1952–53). To be sure, this need not be all-trans; it could equally well represent some constant mixture of stereoisomers, in which presumably the all-trans form predominates.

It is tempting to suppose that the formation of rhodopsin may involve a comparable mechanism—the formation of a halochromic retinyl ion to which is due both the color of rhodopsin and the eventual isomerization to the all-trans configuration. The analogy between rhodopsin and the antimony chloride product is strengthened by the recent observation that the latter is highly sensitive to light (Wald, 1947–48). In this view of rhodopsin structure its prosthetic group is formed of a single molecule of retinene. This is in good agreement with our demonstration that the synthesis of rhodopsin follows a bimolecular course when retinene and opsin are present in approximately equal amount; and seems to become monomolecular when opsin is in large excess (Chase and Smith, 1939–40; see our footnote 13, page 292).[17]

There is enough that is attractive in such a hypothesis to make it worth careful examination. The analogy of rhodopsin with the antimony chloride product of retinene, however, also involves a discrepancy. As already noted, the antimony chloride reaction is in accord with Meunier's hypothesis in that all the isomers of retinene yield the same, possibly all-trans product. The active isomers of retinene yield with opsin, however, two different products, rhodopsin and isorhodopsin, both apparently still cis. Therefore either the latter are not halochromic structures, or Meunier is mistaken in believing that such structures demand the all-trans configuration.

General Considerations.—The experiments we have described introduce a new factor in the biochemistry of vitamin A—that of stereochemical specificity in its reactions with enzymes and other proteins. Certain of its reactions involve this factor acutely, others relatively little. We have already encountered an example of each kind: high specificity in the reaction of retinene with opsin,

[17] There are interesting similarities between rhodopsin regarded in this way and crustacyanin, the blue astaxanthin-protein of the lobster shell (Wald, Nathanson, Jencks, and Tarr, 1948). Crustacyanin possesses a main absorption band at about 637 mμ. On denaturation it "bleaches" to a mixture of red astaxanthin, with $\lambda_{max.}$ at 495 mμ, and colorless protein. Kuhn and Sörensen (1938) suggested that the blue pigment represents a complex of an astaxanthin anion with protein—again, therefore, a halochromic structure. We have found incidentally that the astaxanthin which emerges from crustacyanin on denaturation is in the all-trans configuration (Wald and Jencks, unpublished observations).

relative indifference in the reaction of retinene or vitamin A with alcohol dehydrogenase. This type of relation will need to be considered in all future work on vitamin A metabolism.

It is important, however, to distinguish such biochemical relations from the nutritional behavior of vitamin A. Since vitamin A isomerizes in the body, no high order of isomer specificity is to be expected nutritionally. It has already been shown that in the rat all-trans vitamin A is slightly more potent than neovitamin A*a* (Harris *et al.*, 1951); and larger differences in nutritional effectiveness have been demonstrated by Zechmeister, Deuel, and their colleagues among certain cis-trans isomers of the carotenoid precursors of vitamin A (Zechmeister, 1949). Such differences may have very complicated causes: differences in stability, absorption from the intestine, ease of transport in the blood, and permeability into the tissues, as well as stereochemical specificity in enzyme reactions. In time all such differences are probably obliterated by stereoisomerization. It is probably true therefore that the inclusion of any geometrical isomer of vitamin A in the diet fulfills with greater or less efficiency all the nutritional needs for this vitamin.

APPENDIX

Materials and Methods

Opsin and rhodopsin were prepared from the rods of cattle retinas by methods described previously (Wald and Brown, 1951–52). The outer segments of the rods are separated from the remaining retinal tissues by differential centrifugation; are tanned with alum to make other proteins than rhodopsin insoluble; leached with water and buffer solutions to remove other water-soluble substances; frozen-dried and extracted with petroleum ether to remove lipids; and finally extracted with 2 per cent digitonin (Hoffmann-LaRoche), which brings opsin and rhodopsin into aqueous solution. In the case of rhodopsin all these procedures are carried out in dim red light, in the case of opsin in daylight.

Crystalline alcohol dehydrogenase, prepared from horse livers, was generously supplied by Dr. R. K. Bonnichsen of the Biochemical Divison of the Medical Nobel Institute in Stockholm, Sweden (Bonnichsen, 1950).

Cozymase (DPN) was purchased from the Sigma Company of New York in the "90 per cent pure" grade. *Reduced cozymase* was prepared by the method of Ohlmeyer (1938) from cozymase obtained from Nutritional Biochemicals, Inc., of Cleveland, Ohio.

Retinene for these experiments was prepared by the chromatographic oxidation of vitamin A on manganese dioxide, modified slightly from the method described by Wald (1947–48). As solvent for the oxidation, in place of the petroleum ether used originally, we used 1 to 2 per cent ethyl alcohol in petroleum ether. Mr. Paul Brown in our laboratory has found that this mixture yields considerably increased amounts of retinene with no loss of purity. The product is transferred to alcohol-free petroleum ether, and is further purified by adsorption on a column of calcium carbonate. A small quantity of yellow contaminants is adsorbed at the top of the column. The retinene travels downward at a moderate rate, and is collected in the filtrate.

Hydroxylamine (1 M) was freshly prepared by bringing the hydrochloride, in water solution, to pH about 6.2 with sodium hydroxide.

Synthesis of Rhodopsin.—The method of testing for the synthesis or regeneration of rhodopsin has been described earlier (Wald and Brown, 1951–1952). All measurements were made with the Beckman DU spectrophotometer, the cell compartment of which was maintained at an even temperature by circulating water from a thermostatted bath. Micro absorption cells were used, 1 cm. in depth and 3 mm. wide, holding 0.7 ml. The reaction mixtures were generally made up in a volume of 0.5 ml. The rise and fall of rhodopsin concentration were followed by periodic measurements at 500 mμ, its absorption maximum. Such measurements are not wholly reliable in themselves, since retinene enters into other types of combination with opsin and other molecules, some of which absorb appreciably at 500 mμ, and some of which are light-sensitive. For this reason the product was always tested finally by adding hydroxylamine (0.1 ml. of 1 M solution), which we have found destroys such fortuitous complexes by itself combining with retinene to form retinene oxime, leaving rhodopsin unaltered (Wald and Brown, 1950).

Difference Spectra.—After the addition of hydroxylamine, the absorption spectrum was measured in the dark, and again after exhaustive bleaching in the light. The difference in absorption before and after bleaching, the so called difference spectrum, identifies rhodopsin and measures its quantity.

When rhodopsin is bleached without hydroxylamine, the resulting mixture of retinene and opsin, with $\lambda_{max.}$ at about 385 mμ, has appreciable absorption in the region of the rhodopsin maximum, which distorts the difference spectrum. In the presence of hydroxylamine, the retinene liberated in bleaching is converted immediately to the oxime, with $\lambda_{max.}$ at about 365 mμ, and no appreciable absorption at 500 mμ. Under these conditions the maximum of the difference spectrum is identical in position and height with that of the absorption spectrum.

Determination of Retinene Activity.—As a measure of activity we used the capacity of a retinene preparation to form rhodopsin when incubated with excess opsin. A 100 per cent active preparation yields an extinction of rhodopsin equal to the extinction of retinene employed, when both are measured at their absorption maxima in the same volume of digitonin solution. This is a rough index, since the molar extinctions of the various isomers of retinene differ considerably; but it is adequate to the present purpose. In these terms, the activity of a retinene preparation is characterized by the ratio, extinction of rhodopsin formed divided by extinction of retinene employed, expressed as a percentage.

SUMMARY

Vitamin A and retinene, the carotenoid precursors of rhodopsin, occur in a variety of molecular shapes, cis-trans isomers of one another. For the synthesis of rhodopsin a specific cis isomer of vitamin A is needed. Ordinary crystalline vitamin A, as also the commercial synthetic product, both primarily all-trans, are ineffective.

The main site of isomer specificity is the coupling of retinene with opsin. It is this reaction that requires a specific cis isomer of retinene. The oxidation of vitamin A to retinene by the alcohol dehydrogenase–cozymase system displays only a low degree of isomer specificity.

Five isomers of retinene have been isolated in crystalline condition: all-trans; three apparently mono-cis forms, neoretinenes *a* and *b* and isoretinene *a*; and one apparently di-cis isomer, isoretinene *b*. Neoretinenes *a* and *b* were first isolated in our laboratory, and isoretinenes *a* and *b* in the Organic Research Laboratory of Distillation Products Industries.

Each of these substances is converted to an equilibrium mixture of stereoisomers on simple exposure to light. For this reaction, light is required which retinene can absorb; *i.e.*, blue, violet, or ultraviolet light. Yellow, orange, or red light has little effect. The single geometrical isomers of retinene must therefore be protected from low wave length radiation if their isomerization is to be avoided.

By incubation with opsin in the dark, the capacity of each of the retinene isomers to synthesize rhodopsin was examined. All-trans retinene and neoretinene *a* are inactive. Neoretinene *b* yields rhodopsin indistinguishable from that extracted from the dark-adapted retina ($\lambda_{max.}$ 500 mμ). Isoretinene *a* yields a similar light-sensitive pigment, *isorhodopsin*, the absorption spectrum of which is displaced toward shorter wave lengths ($\lambda_{max.}$ 487 mμ). Isoretinene *b* appears to be inactive, but isomerizes preferentially to isoretinene *a*, which in the presence of opsin is removed to form isorhodopsin before the isomerization can go further.

The synthesis of rhodopsin in solution follows the course of a bimolecular reaction, as though one molecule of neoretinene *b* combines with one of opsin. The synthesis of isorhodopsin displays similar kinetics.

The bleaching of rhodopsin, whether by chemical means or by exposure to yellow or orange (*i.e.*, non-isomerizing) light, yields primarily or exclusively all-trans retinene. The same appears to be true of isorhodopsin. The process of bleaching is therefore intrinsically irreversible. The all-trans retinene which results must be isomerized to active configurations before rhodopsin or isorhodopsin can be regenerated.

A cycle of isomerization is therefore an integral part of the rhodopsin system. The all-trans retinene which emerges from the bleaching of rhodopsin must be isomerized to neoretinene *b* before it can go back; or if first reduced to all-trans vitamin A, this must be isomerized to neovitamin A*b* before it can regenerate rhodopsin. The retina obtains new supplies of the neo-*b* isomer: (*a*) by the isomerization of all-trans retinene in the eye by blue or violet light; (*b*) by exchanging all-trans vitamin A for new neovitamin A*b* from the blood circulation; and (*c*) the eye tissues may contain enzymes which catalyze the isomerization of retinene and vitamin A *in situ*.

When the all-trans retinene which results from bleaching rhodopsin in orange or yellow light is exposed to blue or violet light, its isomerization is accompanied by a fall in extinction and a shift of absorption spectrum about 5 mμ toward shorter wave lengths. This is a second photochemical step in the bleaching of rhodopsin. It converts the inactive, all-trans isomer of retinene into a mixture

of isomers, from which mixtures of rhodopsin and isorhodopsin can be regenerated.

Isorhodopsin, however, is an artefact. There is no evidence that it occurs in the retina; nor has isovitamin A*a* or *b* yet been identified *in vivo*.

In rhodopsin and isorhodopsin, the prosthetic groups appear to retain the cis configurations characteristic of their retinene precursors. In accord with this view, the β-bands in the absorption spectra of both pigments appear to be cis peaks. The conversion to the all-trans configuration occurs during the process of bleaching.

The possibility is discussed that rhodopsin may represent a halochromic complex of a retinyl ion with opsin. The increased resonance associated with the ionic state of retinene might then be responsible both for the color of rhodopsin and for the tendency of retinene to assume the all-trans configuration on its release from the complex.

A distinction must be made between the immediate precursor of rhodopsin, neovitamin A*b*, and the vitamin A which must be fed in order that rhodopsin be synthesized *in vivo*. Since vitamin A isomerizes in the body, it is probable that any geometrical isomer can fulfill all the nutritional needs for this vitamin.

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