RETINENE ISOMERASE

By RUTH HUBBARD*

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

(Received for publication, December 21, 1955)

The Isomerase Effect

Rhodopsin is synthesized by the combination of opsin with a cis isomer of retinene, called neo-*b*; and bleaches to a mixture of opsin and all-trans retinene (Hubbard and Wald, 1952–53). The latter must be re-isomerized to neo-*b* before it can contribute again to rhodopsin synthesis. For vision to go on, therefore, all-trans retinene—or the all-trans vitamin A with which it is in equilibrium—must be continuously isomerized to neo-*b* (*viz.* Fig. 1). This is the process with which the present paper is concerned.

When a light-adapted animal is placed in the dark, the rhodopsin concentration rises immediately in an essentially linear fashion, and levels off gradually, reaching the dark-adapted level in about 3 hours in the frog (Zewi, 1939) and in about 1 hour in man and the rabbit (Rushton *et al.*, 1955). A supply of neo-*b* retinene is therefore available. What is its source?

There is as yet no indication that neo-*b* vitamin A occurs outside the eye.[1] Large stores of it have, however, been found in the eyes of the lobster (Wald and Burg, 1955), and several workers in this laboratory have identified neo-*b* vitamin A in retinas and pigment layers of cattle and frogs.[2] It appears likely that the eye itself possesses a mechanism for producing neo-*b* from all-trans retinene or vitamin A.

There have been indications that all-trans retinene or vitamin A can, at

* This research was supported in part by grants to Professor George Wald from the United States Public Health Service (Grant Number B-568C), and The Rockefeller Foundation. An abstract has appeared elsewhere (Hubbard, 1955). I should like to thank Professor Wald for many helpful discussions.

[1] In a series of preliminary experiments on the distribution of neo-*b* vitamin A, Mrs. P. S. Brown in this laboratory has found it only in the eye (retina and pigment layers). We have previously reported the formation of "rhodopsin" using a fish liver concentrate as the source of vitamin A (Wald and Hubbard, 1950; Hubbard and Wald, 1951). Reexamination of the data shows that we were in fact forming *isorhodopsin* (*cf.* Hubbard and Wald, 1952–53). The liver concentrate therefore contained iso-*a* vitamin A, and not the neo-*b* isomer.

[2] Preliminary experiments show that the neo-*b* isomer constitutes at least 25 per cent of the total vitamin A in these tissues.

J. Gen. Physiol., 1956, Vol. 39, No. 6

least to some extent, act as rhodopsin precursors *in vitro*: (1) Bliss (1951 *a*) prepared saline extracts from frog pigment layers which promoted regeneration in solutions of bleached rhodopsin (containing therefore all-trans retinene and opsin); (2) Hubbard and Wald (1951) described a water-soluble factor from frog pigment layers which promotes regeneration in homogenates of bleached frog retinas; and (3) Collins and co-workers (1953,1954) have reported the regeneration of rhodopsin from all-trans vitamin A in homogenates of rat or cattle retinas, and of frog retinas plus pigment layers.

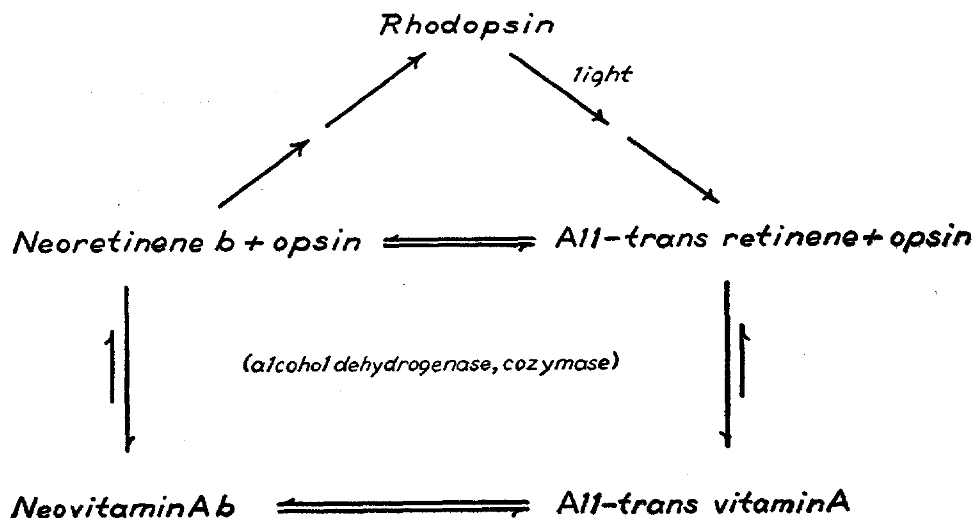


Fig. 1. The rhodopsin cycle. The bleaching of rhodopsin yields all-trans retinene and opsin; the synthesis of rhodopsin requires a cis isomer of retinene, neo-*b*. Both retinenes are in equilibrium with the corresponding isomers of vitamin A. Rod vision therefore depends on the isomerization of all-trans retinene or vitamin A to the neo-*b* isomer.

We have therefore looked for an isomerizing enzyme which might convert all-trans retinene or vitamin A to the neo-*b* isomer. Preliminary experiments[3] showed that buffer or saline extracts of cattle retinas or frog pigment layers promote rhodopsin synthesis in solution in a system containing either all-trans retinene and opsin, or all-trans vitamin A, alcohol dehydrogenase, DPN, and opsin. In both cases opsin must be present throughout the reaction to trap the neo-*b* retinene (*cf.* Hubbard and Wald, 1951). The activity of such extracts was destroyed by heating, and reduced in the presence of iodoacetamide. It is due to an enzyme which we have called *retinene isomerase.* Rho-

[3] The early experiments were carried out by Dr. R. I. Gregerman, whom we wish to thank also for many helpful discussions.

dopsin synthesis in the presence of the isomerase however, was slow; the yields were poor even after 15 hours of incubation, and were not improved by the addition of a number of possible cofactors such as ATP, Mg^{++}, glutathione, or cysteine.

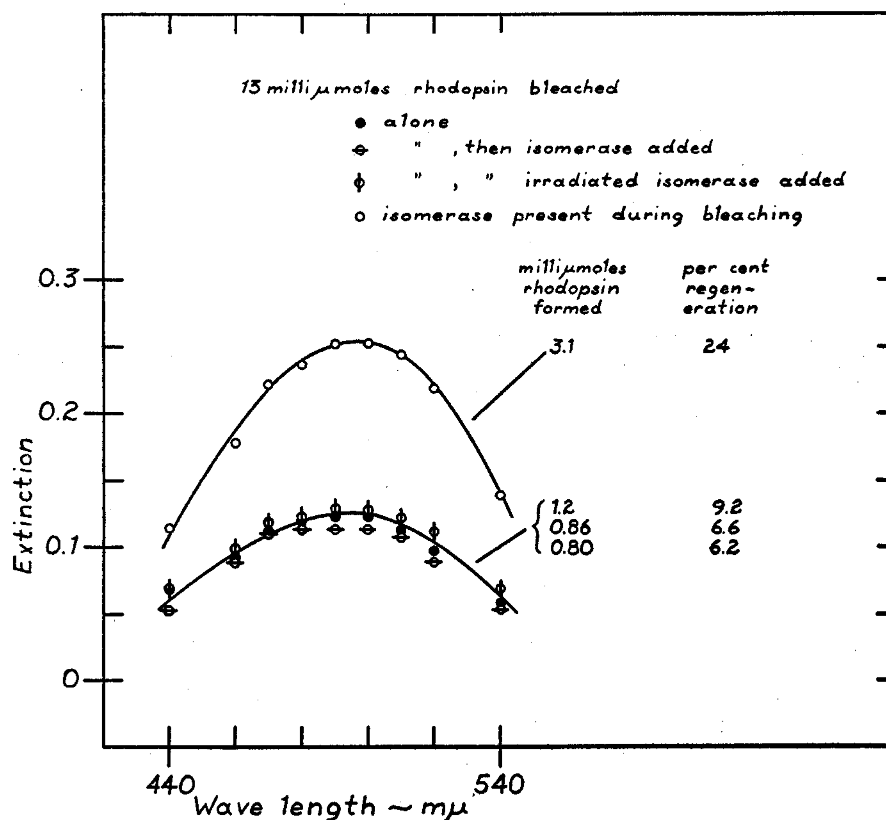


FIG. 2. Regeneration of rhodopsin after bleaching. Rhodopsin was bleached alone and in the presence of isomerase, and then allowed to regenerate. The control regenerated 7 per cent of the rhodopsin, the experimental 24 per cent. Isomerase is effective only when present during bleaching. Addition of isomerase at the end of bleaching, did not raise the regeneration above the control level. Irradiation of isomerase by itself has no effect.

In order to mimic the physiological situation more closely we decided to bleach rhodopsin in the presence of the isomerase. And the results here were more encouraging, as about one-fourth to one-third the all-trans retinene liberated on bleaching was converted to the neo-*b* isomer. Addition of isomerase to bleached rhodopsin resulted only in the slow synthesis of rhodopsin men-

tioned above. Irradiation of the isomerase alone did not enhance its effectiveness. Rhodopsin therefore must be bleached *in the presence of the isomerase* in order to yield appreciable amounts of neo-*b* retinene. Such an experiment is illustrated in Fig. 2.

When rhodopsin is exposed to light at neutral pH, it is first converted to orange products (lumi- and meta-rhodopsin) and finally to all-trans retinene and opsin (Wald, 1937–38; Wald, Durell, and St. George, 1950). This reaction sequence is completed in about half an hour at room temperature. The experiments cited above do not distinguish whether the orange intermediates or all-trans retinene itself are the substrates for this photoisomerization.

TABLE I

Regeneration of Rhodopsin Irradiated in Presence of Isomerase

12.6 millimicromoles of bleached rhodopsin were irradiated for 4 minutes in the presence of isomerase at various times after bleaching (samples *b*, *c*, and *d*). Sample *a* was irradiated without the isomerase and served as control. All four were then incubated in the dark to allow the opsin to combine with neo-*b* retinene formed during the irradiation. Time is reckoned from the beginning of the initial bleach. The amount of rhodopsin is given as millimicromoles and as per cent of the rhodopsin content before bleaching.

Sample	Time of irradiation	Isomerase	Rhodopsin formed	
			millimicromoles	*per cent*
a	7th to 11th minute	−	1.02	8.1
b	7th " 11th "	+	1.74	13.8
c	16th " 20th "	+	2.29	18.2
d	30th " 34th "	+	2.79	22.2

Table I shows the results of an experiment performed to settle this point. It is clear that irradiation in the presence of isomerase was less effective immediately following exposure to light than at later stages of bleaching. The extent of isomerization in fact parallels the release of all-trans retinene, indicating that retinene is the substrate for the isomerase-catalyzed photoisomerization.

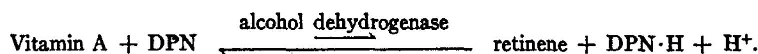
This is shown conclusively by irradiating all-trans retinene in the presence of isomerase, and then incubating the mixture in the dark with excess opsin to test for the neo-*b* isomer. Fig. 3 shows the results of such an experiment performed in the presence of different amounts of isomerase.

Experiment.—Seven samples of all-trans retinene containing 13 millimicromoles each, were mixed with varying amounts of isomerase—from 0.0012 ml. to 0.1 ml. of a stock solution—at pH 7. They were irradiated with orange light for 15 minutes, and incubated with 6.5 millimicromoles of opsin for about 3 hours. (We chose a 3 hour incubation with opsin since this allows sufficient time for opsin to combine with all the neo-*b* retinene formed during irradiation, but is too short for appreciable con-

tamination with rhodopsin formed as a result of the slow dark reaction discussed above.) Hydroxylamine was then added to each sample, and the difference spectra measured. These are shown in Fig. 3, together with a plot of the amount of rhodopsin formed as a function of the isomerase concentration. Isomerase is measured in arbitrary units such that 0.001 ml. of the isomerase preparation is equivalent to 1 unit. Rhodopsin concentration is plotted as millimicromoles. It is equivalent, mole for mole, with the neo-*b* content of the samples, since one molecule of neo-*b* retinene yields one molecule of rhodopsin (*cf.* Hubbard, 1953–54).

As shown in Fig. 3 the yield of neo-*b* retinene increases with isomerase concentration up to about 20 units. Adding more isomerase has little effect. At this point about 37 per cent of the retinene has been converted to neo-*b*.

Irradiation in the presence of the isomerase is effective also with vitamin A, provided that alcohol dehydrogenase and DPN are present. Under these circumstances, a very low concentration of retinene is maintained (*cf.* Bliss, 1951 *b*), and, what is more important, retinene is constantly turned over by the reaction:



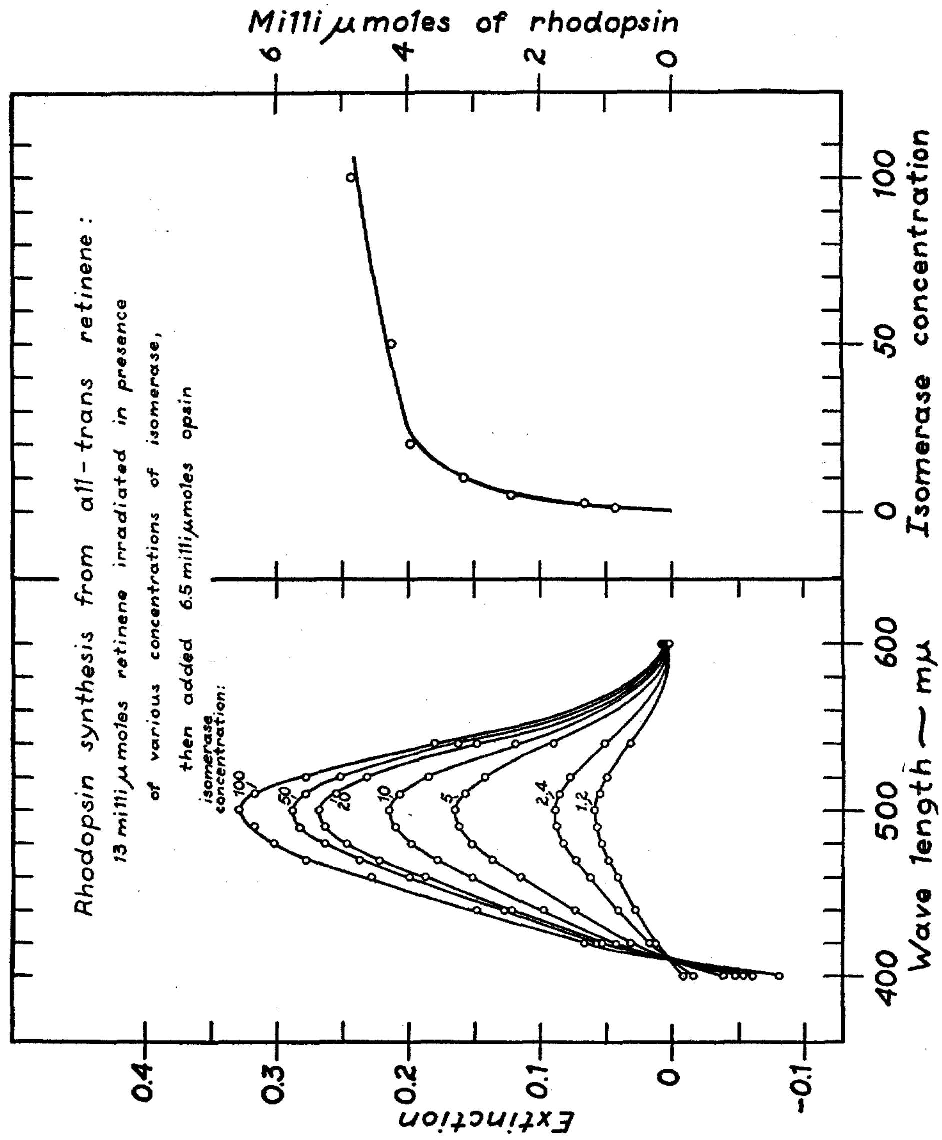
We have irradiated such mixtures with orange light (*i.e.* light absorbed by retinene, but not by vitamin A) and find that neo-*b* is formed only when isomerase is present. This is shown in Table II.

To summarize, we have extracted from cattle retinas an enzyme, retinene isomerase, which catalyzes the slow isomerization of all-trans retinene to the neo-*b* isomer, when incubated in the dark with opsin to trap the neo-*b* retinene. When all-trans retinene is *irradiated* in the presence of isomerase, about 32 per cent is converted to neo-*b*; and for this process trapping by opsin is not required (*cf.* Table III).

Mode of Action of the Isomerase

The isomerase not only promotes the isomerization of all-trans to neo-*b* retinene in the light, but also catalyzes the reverse reaction in the dark. This was tested by incubating neo-*b* retinene with isomerase in the dark, and measuring the neo-*b* that remained by its capacity to form rhodopsin. In one experiment, this was halved in 10 minutes and reduced to less than 10 per cent in an hour, while the *total* retinene concentration—irrespective of configuration—had fallen only slightly. Spectroscopic examination of the retinene absorption band showed a rise in extinction and a slight shift of maximum toward longer wave lengths, indicating the isomerization of neo-*b* to all-trans.

Table IV summarizes seven experiments in which the dark reaction was allowed to come to equilibrium, starting with either all-trans or neo-*b* retinene and isomerase. The course of the reaction is illustrated in Fig. 4. Table V shows the rate of non-specific destruction of retinene during the incubation.



Rhodopsin synthesis from all-trans retinene:
13 milliμmoles retinene irradiated in presence
of various concentrations of isomerase,
then added 6.5 milliμmoles opsin
isomerase concentration:
100
50
20
10
5
2.4
1.2
Extinction
0.4
0.3
0.2
0.1
0
-0.1
Wave length — mμ
400
500
600
Isomerase concentration
0
50
100
Milliμmoles of rhodopsin
6
4
2
0

Fig. 3. Rhodopsin synthesis as an indication of the formation of neo-*b* from all-trans retinene, irradiated in the presence of various amounts of isomerase. The amount of neo-*b* is determined by adding excess opsin after the irradiation, and incubating in the dark for 3 hours. The difference spectra of the rhodopsin formed are shown in the left-hand portion of the figure, along with the isomerase concentration at which each curve was obtained. From these curves, we calculate the moles rhodopsin formed, which are equivalent to the moles neo-*b* present before the addition of opsin. These values are plotted as a function of isomerase concentration in the right-hand portion of the figure. Isomerase concentration is stated in arbitrary units, 1 unit corresponding to 0.001 ml. of a stock isomerase solution.

Experiment.—Fig. 4 illustrates an experiment in which neo-*b* and all-trans retinene, respectively, were incubated in neutral M/15 phosphate buffer at 36°C. by themselves, and in the presence of isomerase. Each sample contained 31.6 millimicromoles retinene per ml. The entire experiment was carried out in dim red light. Aliquots were removed

TABLE II

Formation of Neo-b during Irradiation of a Mixture of All-Trans Vitamin A, Isomerase, and the Alcohol Dehydrogenase System

Three samples were irradiated with orange light for 1 hour. Each contained 28 millimicromoles all-trans vitamin A in a total volume of 0.8 ml. neutral M/15 phosphate buffer. One contained isomerase, alcohol dehydrogenase, and DPN (250 μg.). The others served as controls, omitting either the isomerase or the alcohol dehydrogenase–DPN mixture, as shown in the table. Following irradiation, all three were assayed for neo-*b* by incubation with opsin and addition of the component omitted during irradiation. Only irradiation of the complete system generated neo-*b*.

Components present during irradiation			Rhodopsin formed
All-trans vitamin A	Isomerase	Alcohol dehydrogenase + DPN	
			millimicromoles
+	+	+	1.42
+	+	−	None
+	−	+	None

TABLE III

Isomerase-Catalyzed Photoisomerization of All-Trans Retinene

All-trans retinene was irradiated in the presence of the isomerase and the amount of neo-*b* assayed with opsin at the end of the photoisomerization. The experiment of June 5, 1954, is shown in detail in Fig. 3; the experiment of August 26, 1954, in Fig. 9.

Date	Time of irradiation	Total amount of retinene	Amount of neo-*b* formed	Isomerization
1954	*min.*	*millimicromoles*	*millimicromoles*	*per cent*
Apr. 10	15	13.6	4.6	34
Apr. 26	60	22.7	6.8	30
June 5	15	13.5	4.9	36
June 8	20	11.9	4.4	37
June 9	15	11.9	3.5	30
July 13	15	14.9	4.1	28
Aug. 26	64 and 128	28.7	8.1	28
Average				32

at the times shown in the figure, chilled on ice to stop the isomerization, and incubated with an excess of opsin for 3 hours at 20°C. The rate of isomerization of neo-*b* is negligible at 0°C., and although it is measurable at 20°C., the condensation of neo-*b* with opsin is much more rapid than the isomerization, so that only a slight error is introduced by performing the opsin assay in presence of the isomerase. Comparison of the

TABLE IV

Isomerase-Catalyzed Equilibrium between All-Trans and Neo-b Retinene

All-trans or neo-*b* retinene was incubated with isomerase in the dark. Initially, and at various times during the incubation, the neo-*b* content was determined by assay with opsin. The table shows the total amount of retinene (whether all-trans or neo-*b*) present at the beginning of the incubation, and the amount of neo-*b* at equilibrium. The composition of the equilibrium mixture is described also in terms of per cent neo-*b*. The experiments of January 17, 1955 and January 18, 1955 are shown also in Fig. 4. The two experiments of March 15, 1955, were performed at different levels of isomerase, one 5 times the other. Neither retinene nor isomerase concentration affects the composition of the equilibrium mixtures.

Date	Isomer incubated with isomerase	Duration of incubation	Total retinene	Neo-*b* content of equilibrium mixture	
1955		*hrs.*	*millimicromoles*	*millimicromoles*	*per cent*
Jan. 12	Neo-*b*	2 to 6	3.7	0.34	9.2
Jan. 14	All-trans	6	32	1.8	5.6
Jan. 17	Neo-*b*	3	3.1	0.15	4.8
Jan. 18	All-trans	3	12.6	0.3	2.4
Mar. 15	Neo-*b*	2	8.3	0.32	3.9
Mar. 15	Neo-*b*	3	8.3	0.28	3.4
Mar. 25	Neo-*b*	2	5.4	0.24	4.4
Average					4.6

TABLE V

Destruction of Retinene during Incubation with Isomerase

The table lists total retinene concentration as determined at various times during the incubation. The rate of destruction is similar in all five experiments, three performed with neo-*b*, and two with neo-*a*. The two experiments of March 15, 1955, were run at different enzyme concentrations, the second one-fifth of the first, and represent aliquots from the experiment of the same date shown in Table IV.

Date	Isomer	Time of incubation	Total retinene	Retinene destroyed during incubation	
1955		*min.*	*μg.*	*micrograms*	*per cent*
Mar. 15	Neo-*b*	0	12	—	—
		30	11	1	8
		60	10	2	17
		120	9	3	25
Mar. 15	Neo-*b*	0	12	—	—
		60	10	2	17
		120	9	3	25
		180	8.4	3.6	30
Mar. 18	Neo-*a*	0	12.2	—	—
		30	10.2	2.0	16
		60	9.3	2.9	24
Mar. 25	Neo-*b*	0	0.55	—	—
		60	0.41	0.14	25
		120	0.40	0.15	27
Mar. 25	Neo-*a*	0	11.9	—	—
		65	9.8	2.1	18
		125	8.6	3.3	28

initial assays of neo-*b* in Fig. 4 shows that the assay is about 6 per cent lower when isomerase is present. The ordinate in Fig. 4 is expressed as millimicromoles neo-*b* retinene per milliliter of sample. These values were calculated from the rhodopsin difference spectra obtained after addition of hydroxylamine.

Neo-*b* retinene constitutes about 5 per cent of the equilibrium mixture calculated on the basis of initial retinene concentration (*viz.* Table IV and Fig.

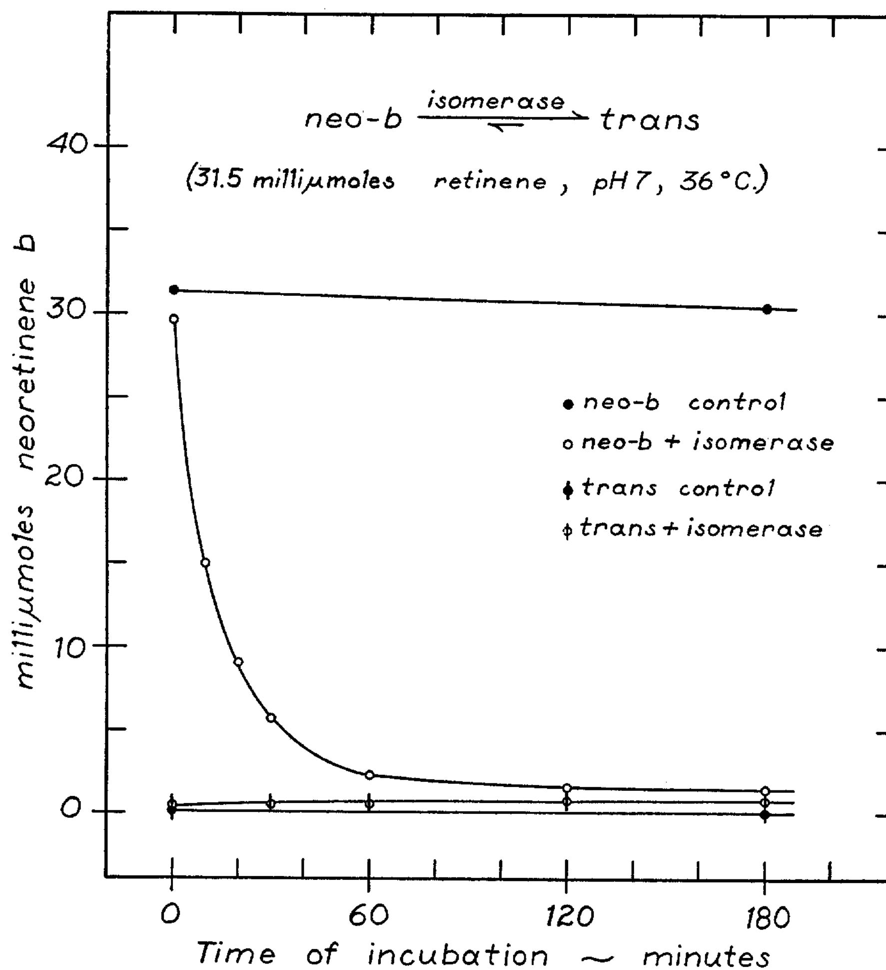


FIG. 4. The isomerase-catalyzed equilibrium between all-trans and neo-*b* retinene. Either isomer (31.5 millimicromoles) was incubated with isomerase in the dark and periodically assayed with opsin for neo-*b* retinene. The two experimental samples came to equilibrium when they contained 0.74 and 1.5 millimicromoles neo-*b*, respectively; *i.e.*, 2 to 5 per cent of the total retinene concentration.

4). Since about 25 per cent of the retinene is destroyed during the incubation (Table V), the actual neo-*b* content of the isomerate is slightly higher. All these reactions were allowed to come to equilibrium in the dark, without an external supply of energy. It is therefore likely that this is the thermodynamic equilibrium, in contrast with the pseudoequilibrium mixtures produced in the light.[4]

Some Properties of the Isomerase Reaction

pH Dependence.—The pH activity curve of the enzymatic isomerization of neo-*b* retinene is shown in Fig. 5. The isomerase activity rises gradually up to pH 10, then levels off. By pH 11, it has not begun to drop. The high pH itself does not isomerize neo-*b*, as shown by the control curve. Our experiments are usually performed near pH 7, far from the pH optimum. This was done so that we could study the isomerase-catalyzed *light* reaction at a physiological pH, and compare it with the *dark* isomerization under identical conditions of pH and enzyme concentration.

Experiment.—Aliquots of isomerase in neutral M/15 phosphate buffer were brought to various pH's. All operations were performed in dim red light. The isomerase samples were mixed with neo-*b* retinene (24 millimicromoles) and incubated for 7 minutes at 36.5°C. A 7 minute incubation yields an approximate measure of the rate of reaction, since the rate up to this time is roughtly constant (*cf.* Fig. 4). The samples were then chilled on ice to stop the reaction, and neutralized when necessary. Aliquots of each sample containing 8 millimicromoles of retinene were now mixed with 11 millimicromoles of opsin, and the assay mixtures incubated in the dark for 3 hours at 20°C. Fig. 5 shows the results of three sets of experiments, each covering the entire pH range; the individual points represent data from single samples.

Dependence of Rate on the Concentration of Neo-b Retinene and Isomerase.—Fig. 6 shows the results of a series of experiments performed at different levels of enzyme and substrate, and plotted in terms of the rate of reaction against substrate concentration. By "rate" we mean the amount of neo-*b* retinene isomerized in 10 minutes. This is a rough estimate of the initial rate at low levels of enzyme (*cf.* Fig. 4). At the higher enzyme concentrations, a 10 minute incubation is too long for a determination of initial rate, and therefore yields less sharp substrate-saturation curves. All four curves show an initial increase in rate with substrate concentration; but only in the experiments performed with 5 and 10 units of enzyme does the rate level off at the highest substrate

[4] Mixtures of isomers produced by irradiation of carotenoids have sometimes been referred to as isomer "equilibria." The production of isomerates by light or heat, however, can involve a gain in the free energy of the system. These mixtures therefore are not states of thermodynamic equilibrium, but "pseudoequilibria" which can vary in composition.

concentrations employed in these experiments. From the two complete saturation curves, we can calculate approximate values of the Michaelis constant (K_M) of the isomerase: the initial rate (as defined above) was half maximal at

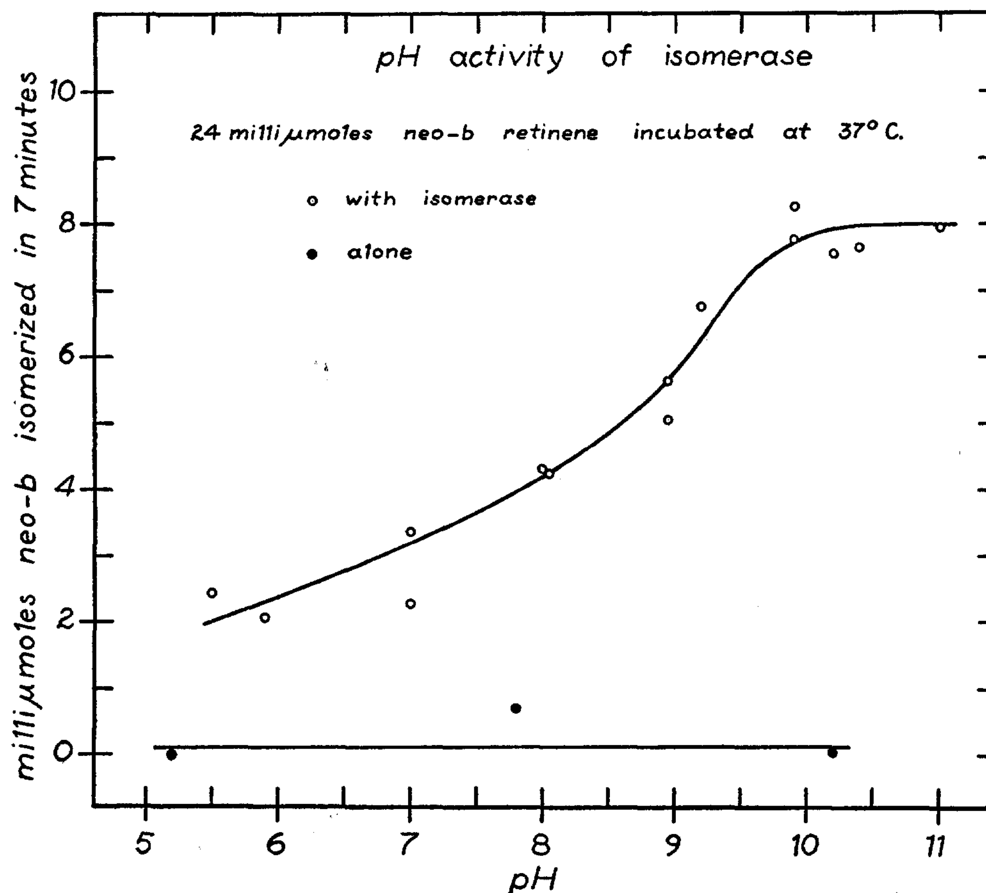


FIG. 5. Effect of pH on isomerase activity. Rate of isomerization of neo-*b* plotted as a function of pH. The isomerase activity increases with pH up to pH 10, then levels off. At pH 11, it has not yet begun to decline. The high pH itself does not isomerize neo-*b*, as shown by the control.

substrate concentrations of about 6 millimicromoles per 0.3 ml. sample. This is equivalent to a K_M of about 2×10^{-5} mole per liter.[5]

[5] Not enough is known about the kinetics of the isomerase system to decide whether the Michaelis-Menten treatment is applicable. This K_M therefore is only a quantitative expression of the fact that the isomerase can be saturated with neo-*b* retinene.

Experiment.—Neo-*b* retinene and isomerase were mixed in the proportions shown in Fig. 6. The isomerase concentration is expressed again in arbitrary units such that 0.001 ml. of an isomerase solution is defined as containing 1 unit. The isomerase was dissolved in neutral M/15 phosphate buffer; the neo-*b* in 1 per cent digitonin. Samples were prepared in dim red light and incubated at 37°C. The reaction was stopped 10 minutes after mixing by chilling the samples on ice and then adding an excess of opsin

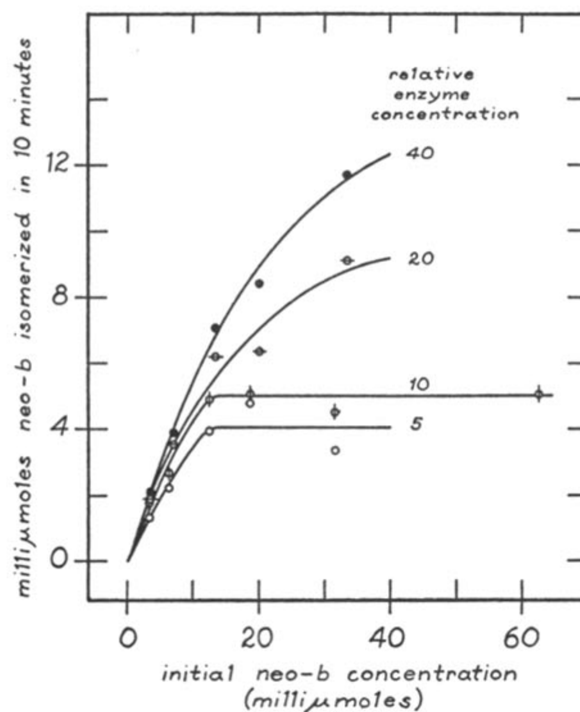


FIG. 6. Rate of isomerization of neo-*b* retinene as a function of the concentration of neo-*b* and isomerase. The rate increases linearly with substrate concentration, then levels off. At the higher enzyme concentrations, the rate only begins to level off within the range of substrate concentrations employed in this experiment.

to assay for neo-*b* retinene. The opsin assays were performed in the dark at 20°C. After 3 hours the rhodopsin concentration was determined in the usual way.

Temperature Quotient (Q_{10}).—The initial rates of the enzyme-catalyzed isomerization of neo-*b* to all-trans retinene in the dark, and of all-trans to neo-*b* retinene in orange light (under conditions in which the rate was limited by light intensity) have been measured at two temperatures: 26 and 36°C. The rate of isomerization in the dark was roughly doubled at the higher tempera-

ture (Q_{10} = about 2), but the rate of isomerization in the light was not changed (Q_{10} = 1).

Recapitulation.—We have extracted from cattle retinas an enzyme, retinene isomerase, which interconverts all-trans and neo-*b* retinene. When the enzyme

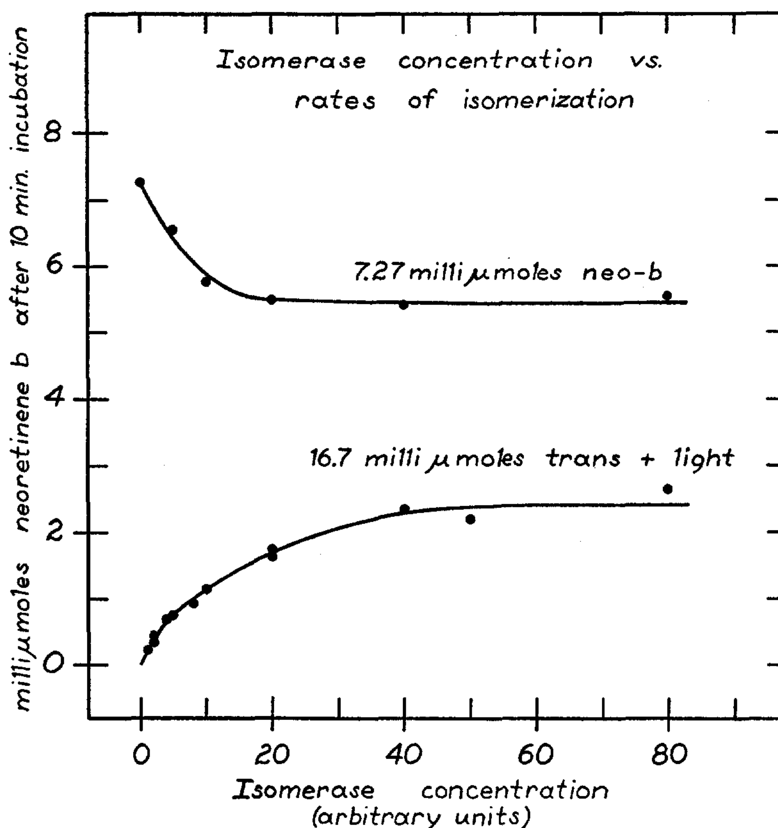


FIG. 7. Rates of the isomerase-catalyzed isomerization of neo-*b* retinene in the dark, and all-trans retinene in the light, as functions of the isomerase concentration. The rates increase with isomerase concentration up to a saturating value. Further increase in isomerase has no effect on the rate of reaction. Either method is used as routine to assay the activity of isomerase preparations (*cf.* Table VII).

is incubated with either isomer in the dark, an equilibrium mixture is formed containing about 5 per cent neo-*b*. If opsin is added during the incubation, it traps the neo-*b* retinene and forms rhodopsin. The enzyme can therefore catalyze the slow synthesis of rhodopsin from all-trans retinene in the dark. If all-trans retinene is incubated with the isomerase in the light, a pseudo-equilibrium is formed which contains about 32 per cent neo-*b*.

These two types of reaction are summarized in Fig. 7 which shows the rates of the enzyme-catalyzed isomerization of neo-*b* retinene in the dark, and of all-trans retinene in the light, as functions of the isomerase concentration. The rate of either reaction increases with enzyme concentration up to a saturating concentration, and then levels off. At this point the rate is no longer limited by the availability of enzyme, but by the substrate concentration, or in the case of the photoisomerization, by substrate and light intensity. It may be significant that in both experiments the ratio of substrate to enzyme at half-saturation is of the same order of magnitude—1.5 to 2.5 millimicromoles retinene per unit of enzyme. Both reactions therefore appear to have approximately the same Michaelis constant.

Experiment.—Various amounts of isomerase were incubated either with 7.27 millimicromoles neo-*b* or 16.7 millimicromoles all-trans retinene. The neo-*b* experiment was carried out in dim red light. The samples were incubated in M/15 phosphate buffer, pH 6.9, at a temperature of 36°C. The incubation was stopped after 10 minutes by chilling, followed by addition of opsin. For the photoisomerization experiment, the mixtures of all-trans retinene and isomerase were also made up in M/15 phosphate buffer, pH 6.9, and the samples irradiated for 10 minutes with an orange light. (The conditions of irradiation were the same as in the experiment shown in Fig. 3.) Opsin was added immediately following irradiation, and the mixtures were left in the dark for 2½ hours at 20°C. Hydroxylamine was then added and the difference spectra measured. The amount of neo-*b* retinene present at the end of the 10 minute incubations is plotted in Fig. 7 as a function of the isomerase concentration, which is again expressed in arbitrary units such that 0.001 ml. of an isomerase preparation contains 1 unit. This type of experiment is performed as routine to assay the isomerase activity of various preparations (*cf.* Table VII).

Substrate Specificity of the Isomerase

Isomers of Retinene.—We have presented evidence that the isomerase catalyzes the interconversion of all-trans and neo-*b* retinene. Retinene, however, occurs also in several other isomeric forms (*cf.* Hubbard, Gregerman, and Wald, 1952–53; Robeson *et al.*, 1955). These are summarized in Fig. 8.[6] The question therefore arises whether the isomerase is specific for neo-*b* or whether it catalyzes also the interconversion of all-trans retinene and other cis forms.

The only isomer of retinene for which we have a convenient assay aside from neo-*b*, is iso-*a*, the precursor of isorhodopsin. The absorption spectra of

[6] We have previously assigned the 7-cis configuration to the neo-*b* isomer of retinene and vitamin A (Wald, Brown, Hubbard, and Oroshnik, 1955). The argument was based in part on the synthesis of an 11-cis isomer of vitamin A (neo-*c*) believed to be *mono-cis*. Reexamination, however, has shown that neo-*c* is in fact the 11,13-*di-cis* isomer. The 11-*mono-cis* isomer has now been synthesized and is indistinguishable in its properties from neo-*b* (Oroshnik, 1956).

rhodopsin and isorhodopsin are sufficiently different to be readily distinguishable, and mixtures of the two can be analyzed from the shape of the difference spectrum. This method allows accurate determination of millimicromole quantities of either isomer in the presence of a fiftyfold excess of the other (for details, see Appendix).

Fig. 9 shows the effect of the isomerase on the rates of formation of iso-*a* and neo-*b* from all-trans retinene. In this experiment all-trans retinene was irradiated with a white light of sufficient intensity to yield an easily measurable rate of *non-enzymatic* photoisomerization, allowing us to compare quantitatively the rates of photoisomerization in the presence and absence of the enzyme. Without isomerase neo-*b* was formed about 8 times as fast as

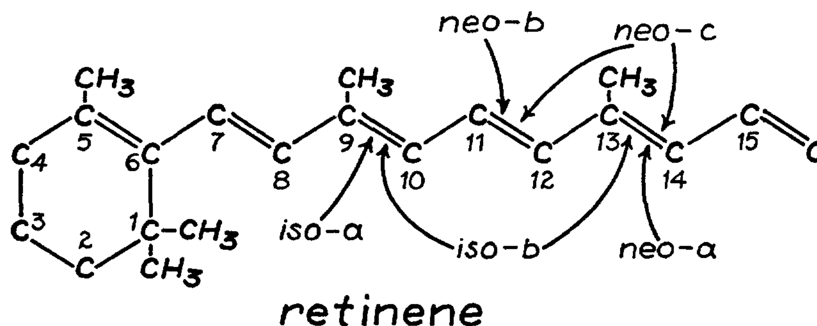


Fig. 8. The structure of retinene. The structure as drawn has the all-trans configuration. Arrows indicate the double bonds which are in cis linkage in the various cis isomers.[6]

iso-*a* and reached a pseudoequilibrium concentration of 15 per cent. The concentration of iso-*a* continued to increase until the experiment was discontinued after 2 hours. This discrepancy in the rates of formation of neo-*b* and iso-*a* is encountered in all experiments involving photoisomerization.

The addition of isomerase increased the rate of formation of neo-*b* about 5 times, and a new pseudoequilibrium was established containing 28 per cent neo-*b*. The rate of formation of iso-*a*, however, was not affected by the isomerase, and both sets of data lie on the same line. The isomerase therefore increases the rate of formation of neo-*b* from all-trans retinene without exerting any effect on the simultaneous formation of iso-*a*.

Experiment.—Two samples of all-trans retinene in buffered digitonin (pH 6.7) were irradiated with the standard light source shielded by a neutral filter of optical density 1.0. The brightness at the samples was about 200 foot-candles. One sample served as control, the other contained enough isomerase to catalyze the maximal rate of isomerization. Aliquots containing 28.7 millimicromoles of retinene were withdrawn at the times shown in Fig. 9, and each was immediately mixed with an excess of opsin

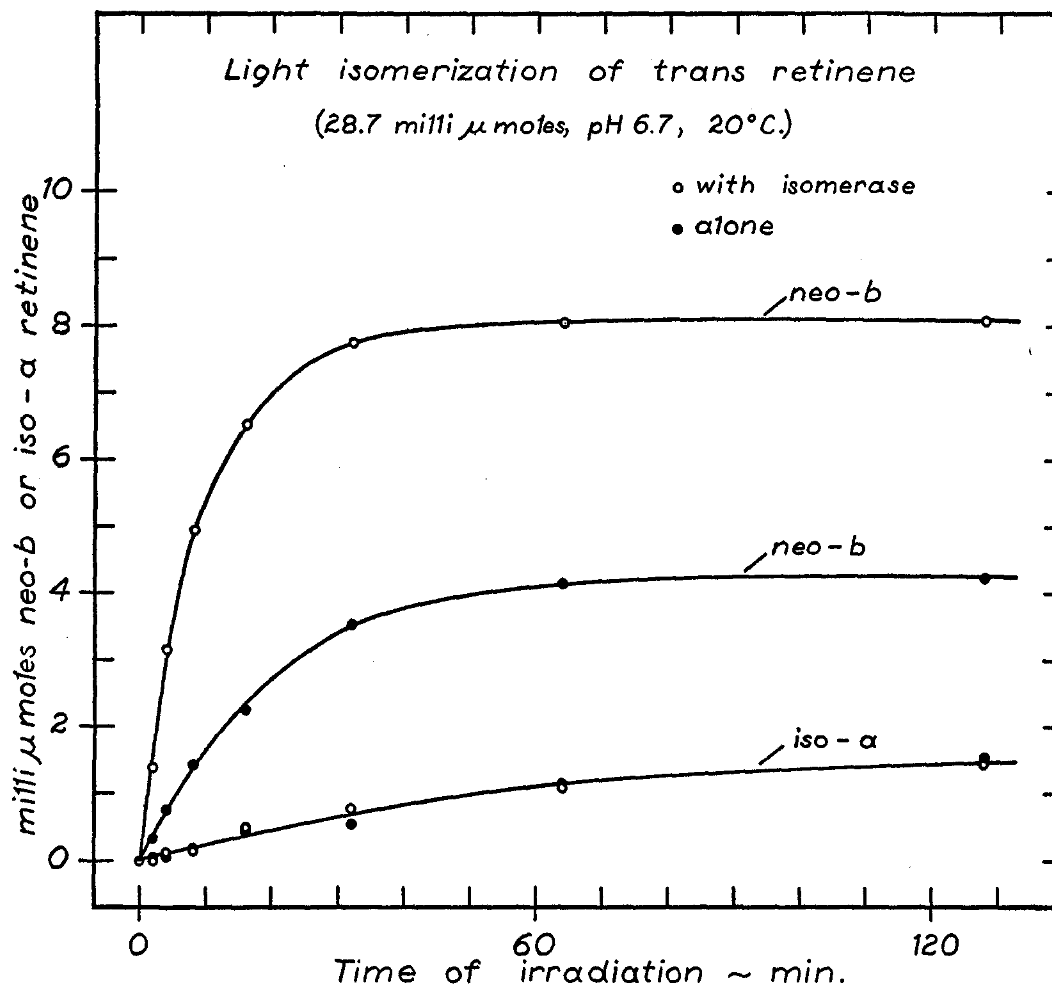


FIG. 9. Formation of neo-*b* and iso-*a* retinenes during the irradiation of all-trans retinene. Without isomerase, neo-*b* retinene is formed about 8 times as fast as iso-*a*. The isomerase increases the initial rate of formation of neo-*b* about 5 times, without exerting any effect on the simultaneous formation of iso-*a*. The pseudoequilibrium concentration of neo-*b* is doubled in the presence of the isomerase.

and incubated in the dark for about 3 hours. Hydroxylamine was then added, and the difference spectra measured. The amounts of rhodopsin and isorhodopsin contained in each were calculated by the method described in the Appendix. These values are plotted along the ordinate and expressed as millimicromoles of neo-*b* or iso-*a* retinene.[7]

[7] I should like to acknowledge the assistance of Mr. Thomas Bibring with the design and execution of this experiment.

Can the isomerase catalyze the interconversion of iso-*a* and all-trans retinene in the dark? We incubated iso-*a* retinene with isomerase in the dark and assayed the amount of iso-*a* present at various times. In parallel, we performed the identical experiment with neo-*b* retinene. The results are shown in Fig. 10 (open circles). While the neo-*b* concentration showed the usual hyperbolic decay curve falling from an initial concentration of 3.83 millimicromoles to 0.57 millimicromole, a decrease of 85 per cent in 40 minutes, the iso-*a* concentration dropped by only about 9 per cent from an initial value of 9.81 millimicromoles to 8.91 millimicromoles. In the absence of the isomerase, the iso-*a* control sample lost 2 per cent iso-*a* retinene during the same period. The isomerase may therefore catalyze a very slow isomerization of iso-*a*, but by comparison with the isomerization of neo-*b*—which in this experiment is half-complete in 8 minutes—the effect on iso-*a* retinene is almost negligible.

Does the presence of iso-*a* affect the isomerization of neo-*b*? In parallel with the above experiments, we prepared a mixture of neo-*b* and iso-*a* retinenes keeping all other conditions the same. The results are also plotted in Fig. 10 (filled circles). The results from the mixed sample are identical with the ones described above. Iso-*a* retinene therefore is neither an effective substrate for the isomerase, nor does it interfere with the isomerization of neo-*b*.

Experiment.—Three samples were prepared containing an excess of isomerase (*i.e.* an amount which was not rate-limiting) in neutral phosphate buffer, and (1) 12.8 millimicromoles neo-*b* retinene per ml., (2) 32.8 millimicromoles iso-*a* per ml., and (3) 12.8 millimicromoles neo-*b* + 32.8 millimicromoles iso-*a* per ml. The controls contained the same amounts of retinene without the isomerase. All six samples (three experimental and three control) were prepared in dim red light and incubated at 37°C. At the times shown in Fig. 10, aliquots were withdrawn, chilled, and incubated with excess opsin at 20°C. After 4 hours, hydroxylamine was added and the difference spectra measured. The method for calculating the amounts of neo-*b* and iso-*a* retinene is described in the Appendix.

A number of years ago, a cis isomer of vitamin A (now called neo-*a*) was isolated from liver oils (Robeson and Baxter, 1947). When the corresponding isomer of retinene is incubated with the isomerase in the dark, virtually no neo-*b* is formed. Some of the neo-*a* retinene is converted to all-trans, but the extent of the conversion at "equilibrium" varies with the isomerase concentration. The isomerization is therefore probably non-specific, owing to the fact that the neo-*a* and all-trans isomers of retinene equilibrate rather easily (*cf.* Ames *et al.*, 1955; Hubbard, 1956).

All-Trans and Neo-b Vitamin A.—We mentioned above that mixtures of all-trans vitamin A, alcohol dehydrogenase, DPN, and opsin in the dark slowly synthesize rhodopsin in the presence of the isomerase. Such experiments, however, cannot distinguish whether the isomerase acts directly on all-trans vitamin A, or on the all-trans retinene which is formed as a result of the oxidation of vitamin A by DPN and alcohol dehydrogenase.

Since the all-trans—neo-*b* equilibrium strongly favors the all-trans isomer, direct spectroscopic examination of the ultraviolet absorption spectrum of neo-*b* vitamin A in presence and absence of the isomerase should reveal whether the isomerase catalyzes the interconversion of these two isomers of vitamin

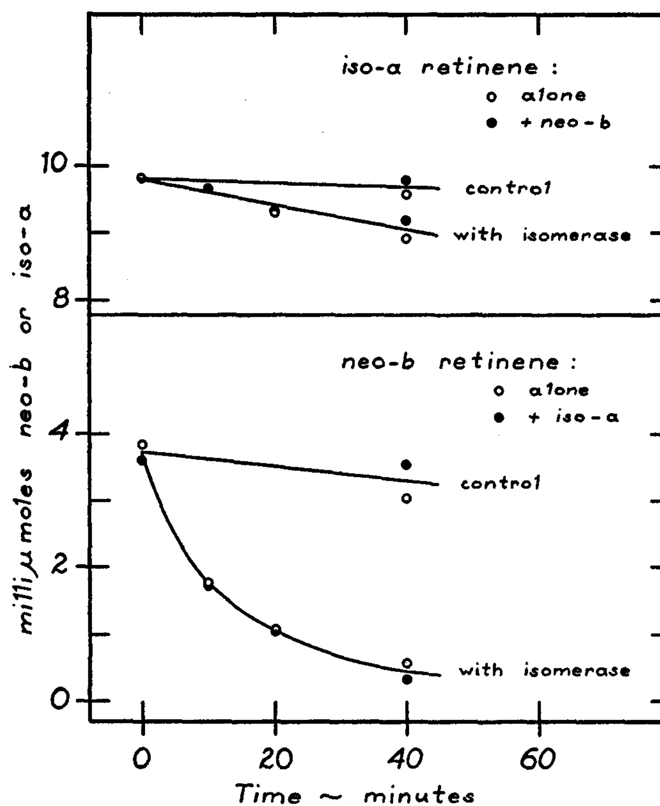


FIG. 10. Isomer specificity of the isomerase. A 40 minute incubation of isomerase with neo-*b* retinene isomerizes 85 per cent to all-trans; in a comparable incubation with iso-*a* retinene, only 9 per cent is isomerized. The rates are unchanged when the isomerase acts on a mixture of neo-*b* and iso-*a*. The isomerase is therefore specific for neo-*b* retinene: iso-*a* does not participate as substrate or inhibitor.

A. The specific extinction of all-trans vitamin A is about 50 per cent higher than that of the neo-*b* isomer (Wald *et al.*, 1955; Brown and Wald, 1956), and its absorption maximum lies at about 328 mμ, while neo-*b* vitamin A absorbs maximally at about 320 mμ. The isomerization of neo-*b* to all-trans vitamin A therefore involves a rise in extinction and a shift of the absorption band towards longer wave lengths.

When neo-*b* vitamin A was incubated with isomerase, neither of these

changes was observed. The data from such an experiment are shown in Table VI. The extinction of the control sample fell by about 18 per cent during the incubation. The extinctions of the experimental samples remained essentially constant. The shape and position of the absorption spectrum were unchanged in control and experimental samples. The fall in extinction of the control sample is probably caused by destruction of vitamin A, and can be prevented by addition of non-specific proteins, such as serum albumin.[8] The isomerase apparently performs the same function in the above experiment. This spectroscopic method is not as sensitive as the opsin assay, and a 10 to 15 per cent isomerization could probably have gone undetected. When the lower of the

TABLE VI

Action of Isomerase on Neo-b Vitamin A

Change in extinction at 320 mμ (the absorption maximum of neo-*b* vitamin A) of three samples containing 4.6 micrograms neo-*b* vitamin A per ml., during incubation for 1 hour at 36.5°C. All samples in neutral M/15 phosphate buffer containing 0.0025 per cent tween 80. In addition, samples *b* and *c* contained isomerase, *c* at twice the concentration of *b*. The data for sample *a* are uncorrected, those for *b* and *c* have been corrected for the extinction of the isomerase preparation at 320 mμ (0.291 and 0.552, respectively).

Time of incubation	Extinction at 320 mμ		
	a without isomerase	*b* with isomerase	*c* with isomerase (twice *b*)
min.			
0	0.552	0.552	0.552
15	0.478	0.552	0.553
30	0.459	0.550	0.554
60	0.450	0.548	0.551

two isomerase concentrations, however, was tested with neo-*b* *retinene*, the reaction came to equilibrium in an hour, and was half-complete in about 8 minutes. The isomerase therefore essentially does not catalyze the isomerization of neo-*b* and all-trans vitamin A.

Recapitulation.—A study of the isomer specificity of the isomerase has shown that it is essentially specific for all-trans and neo-*b* retinene. It does not act on iso-*a* retinene, and probably not on neo-*a* retinene, or on neo-*b* and all-trans vitamin A.

DISCUSSION

Energetics.—When considering the equilibrium between cis and trans isomers of such molecules as retinene and vitamin A, one must bear in mind that the trans compound is thermodynamically the most stable form and there-

[8] This effect was first observed by Dr. N. I. Krinsky in this laboratory.

fore most prevalent in the equilibrium mixture. The relative instability of cis compounds is due to the fact that the cis linkage introduces a slight aplanarity into the molecule (*cf.* Pauling 1939, 1949). Neo-*b* is a *hindered* cis isomer (Robeson *et al.*, 1955; Wald *et al.*, 1955) in which steric hindrance causes a large *twist* in the molecule in addition to the bending introduced by the cis linkage. Such isomers are energetically even less probable than the unhindered cis forms. In fact, it was believed until recently that they are too unstable to occur in isomerates. One would therefore expect the thermodynamic equilibrium between all-trans and neo-*b* retinene to favor the all-trans isomer.

The equilibrium which the isomerase catalyzes in the dark contains about 5 per cent neo-*b* retinene. We have shown that probably no other isomer is involved, so that to a first approximation all-trans retinene constitutes the remaining 95 per cent. An equilibrium mixture of 95 per cent all-trans and 5 per cent neo-*b* implies a difference in free energy (ΔF) of about 2 kcal. per mole between the two isomers, neo-*b* having the higher energy content.

In the light, the isomerase-catalyzed equilibrium is shifted to a ratio of about 68 parts all-trans to 32 parts neo-*b* retinene. Light is clearly supplying free energy to the system. It is also lending activation energy, as the Q_{10} of the enzyme-catalyzed isomerization is shifted from a value of about 2 in the dark to about 1 in the light.

Opsin as an Isomerase.—Opsin also acts as a cis-trans isomerase: it combines with neo-*b* retinene to form rhodopsin, but releases all-trans retinene on bleaching (*cf.* Wald and Brown, 1956). Its specificity, however, is not as rigid as that of the isomerase described above, for opsin combines also with iso-*a* retinene to form isorhodopsin, which on bleaching releases the all-trans isomer (Hubbard, Gregerman, and Wald, 1952–53). Opsin therefore isomerizes both neo-*b* and iso-*a* to all-trans retinene.

The retinenes have their absorption maxima ($\lambda_{max.}$) at about 385 mμ in aqueous solution; quanta of this wave length contain 74 kcal. per mole. Rhodopsin and isorhodopsin have their absorption maxima at 500 and 486 mμ, respectively, corresponding to a quantum energy of about 58 kcal. per mole. Opsin therefore lowers the activation energy for photoisomerization by shifting the absorption spectrum of retinene to longer wave lengths, bringing the action into the visible. In this sense it is comparable with iodine which also catalyzes photoisomerization by rendering smaller quanta effective (*cf.* Zechmeister, 1944). During iodine catalysis, however, the iodine itself absorbs the light, whereas with opsin, retinene still acts as chromophore.

If opsin is an isomerase, then rhodopsin and isorhodopsin are analogous to enzyme-substrate complexes. So regarded, they have the peculiarity that the complex is separated from the reaction product (all-trans retinene) by an activation barrier of about 48 kcal. per mole (*viz.* St. George, 1951–52).

Physiological Correlations.—Rhodopsin synthesis *in vivo* starts as soon as

an animal is replaced in the dark. In the frog, it is complete in about 3 hours (Zewi, 1939); in mammals and man, it is half-complete in about 10 minutes, and complete in an hour (Rushton *et al.*, 1955; Campbell and Rushton, 1955). Can the isomerase reaction account for these phenomena?

We know of three ways of obtaining neo-*b* for dark adaptation: (1) The isomerase-catalyzed photoisomerization of all-*trans* retinene; (2) the slow but steady trapping of neo-*b* by opsin working against the gradient of the isomerase equilibrium in the dark; and (3) the stores of neo-*b* vitamin A (alcohol and ester) in the pigment layers.[2] These stores presumably have to be filled during light adaptation by the isomerase-catalyzed photoisomerization of all-trans to neo-*b* retinene, followed by its reduction to vitamin A.

Two factors mediate against the photoisomerization of retinene in the eye: (1) retinene is isomerized only by light which it can absorb, *i.e.* blue, violet, and ultraviolet; the lens of the eye, however, transmits little light at these wave lengths (Wald, 1945, 1949; Milkman and Kennedy, 1955). Light which reaches the retina therefore is not efficient for the isomerization of retinene.[9] (2) There is very little retinene in the eye: under steady state conditions, retinene is either combined with opsin, as rhodopsin, or reduced to vitamin A by the alcohol dehydrogenase system. It therefore must be isomerized either as it is released from rhodopsin, or during the turn-over of the vitamin A—retinene equilibrium (*viz.* Table II). The isomerase should increase the physiological importance of photoisomerization since it accelerates the rate of isomerization and shifts the equilibrium between all-trans and neo-*b* retinene to a pseudoequilibrium which is considerably more favorable to the neo-*b* isomer.

Granit and coworkers (1938) have performed a series of experiments which support the notion that photoisomerization plays a significant part in dark adaptation. Recording the electroretinogram of the frog after adaptation to colored lights, they found that adaptation with blue or violet caused a smaller lowering of the *b*-wave and a faster recovery than adaptation with green, yellow, or orange lights which had been matched to bleach the same amount of rhodopsin. They concluded that "during adaptation with these wavelengths (*i.e.* blue and violet) a process tending to increase the size of the response to the test light is likewise activated." The spectral sensitivity of this "process" suggests that they were in fact isomerizing retinene.

It is impossible at present to decide whether the isomerase can supply sufficient neo-*b* for dark adaptation. We should like to stress, however, that the rhodopsin cycle is a dynamic system in which the rates of bleaching and synthesizing rhodopsin, and of isomerizing and reducing retinene are balanced against one another, and in which the storage of neo-*b* vitamin A in the pig-

[9] This situation has been exaggerated in the *in vitro* experiments by using yellow or orange light for the isomerase-catalyzed photoisomerization.

ment epithelium may act as a buffer, taking up slack when an excess of neo-*b* is being produced during light adaptation, and giving up its stores as the demand increases during dark adaptation.

Needless to say, there may be alternative mechanisms for producing neo-*b* retinene or vitamin A. There may also be ways of shifting the isomerase-catalyzed equilibrium in the dark by supplying energy in chemical form with an effect analogous to that of light. There is as yet no evidence of such mechanisms, but they would greatly simplify our problem.

APPENDIX

Preparation, Purification, and Assay of the Isomerase.—The extraction and purification of isomerase are carried out in the cold (4°C.). Cattle retinas are dissected on ice and frozen. They can thus be stored for many weeks. For extraction, they are

TABLE VII

Purification of Isomerase. Steps in the Purification of Isomerase Preparations and Relative Purity of the Various Fractions

Total protein is measured by the extinction at 280 mμ, assuming an E (1 per cent, 1 cm.) of 12. Activity is measured by the rate of isomerization of neo-*b* to all-trans retinene, and expressed in arbitrary units, such that 1 ml. of fraction 4 contains 1000 units.

Fraction	Description	Total protein	Total activity	Specific activity
		gm.	*units*	*units per mg.*
1	Crude extract	3.7	46,000	12.5
2	0 to 35 per cent $(NH_4)_2SO_4$ precipitate	0.6	15,000	25
3	0 to 20 per cent $(NH_4)_2SO_4$ precipitate	0.04	2,000	50
4	20 to 35 per cent $(NH_4)_2SO_4$ precipitate	0.2	12,500	62.5

thawed, homogenized with neutral M/15 phosphate buffer (about 2 ml. buffer per retina), and extracted for ½ hour. The suspension is centrifuged at 45,000 to 80,000 times gravity (Spinco preparative ultracentrifuge, Model L, No. 21 or 30 rotors). The supernatant contains the activity (fraction 1, Table VII). It is brought to 35 per cent saturation with ammonium sulfate, and the precipitate separated, dissolved in a small volume of neutral M/15 phosphate buffer, and dialyzed. This solution (fraction 2, Table VII) is refractionated with ammonium sulfate, and the precipitates accumulated between 0 and 20 per cent and between 20 and 35 per cent saturation collected (fractions 3 and 4, Table VII). The precipitates are dissolved in minimum amounts of neutral phosphate buffer and dialyzed. They are centrifuged at 100,000 times gravity (Spinco centrifuge, No. 40 rotor) and the clear solutions drawn off. Fraction 4 has the highest activity and is the fraction used in our experiments. When frozen, the preparation can be stored for many weeks; at 4°C. it slowly loses activity; at 50°C. about two-thirds of the activity is lost in 12 minutes.

The isomerase is assayed by measuring either the rate of isomerization of neo-*b* to all-trans retinene in the dark, or the rate of the reverse reaction in orange light. The

isomerization of neo-*b* to all-trans is carried out at 36–37°C. The photoisomerization is achieved with a 150 watt tungsten filament microscope lamp shielded by Corning filters 3965 and 3484. The brightness of the unfiltered source is about 2000 foot-candles. Both types of assay are illustrated in Fig. 7. To compare the activities of various fractions, we select one as the standard and arbitrarily assign to it an activity of 1000 units per ml. For the fractionation summarized in Table VII, fraction 4 served as standard. A rate curve is constructed with this fraction, plotting rate of isomerization of neo-*b* retinene as a function of isomerase concentration. The other fractions are then assayed at two or three dilutions and the results entered on the rate curve. Each dilution thus yields an estimate of the isomerase concentration; these estimates are averaged. For example, the assays for fraction 1 using 0.0067 ml., 0.0134 ml., and 0.0267 ml. per assay, gave estimates of 194, 172, and 161 units per ml.: an average of 176 units. From the extinction at 280 mμ, we estimated that this fraction contained about 14 mg. of protein per ml. It therefore had a specific activity of 12.5 units per mg. of protein.

Opsin and rhodopsin were prepared by a method similar to the one described by Wald and Brown (1951–52). The retinas are ground with 40 per cent sucrose in neutral M/15 phosphate buffer (1 ml. of sucrose solution per retina) and layered in a plastic centrifuge tube under M/15 neutral phosphate buffer. The suspension is centrifuged at 100,000 times gravity (Spinco preparative centrifuge with No. 40 rotor) for about 15 minutes. The rods float to the sucrose-buffer interface, the retinal debris sediments. The rods are drawn off, and sedimented from buffer. They are then washed with distilled water, hardened in 4 per cent alum (potassium aluminum sulfate) for 10 to 15 minutes, and washed twice with distilled water and once with neutral phosphate buffer. They are now lyophilized and extracted with low boiling petroleum ether. The petroleum ether is evaporated off at room temperature, and the rods are extracted with 2 per cent aqueous digitonin. For rhodopsin, the entire procedure is carried out in dim red light. For opsin, the retinas are dissected under a bright white light and allowed to fade to colorlessness. The fractionation and extraction are then carried out in the light, at about 4°C.

Only crystalline *isomers of retinene* were used. All-trans retinene was prepared by the oxidation of crystalline synthetic all-trans vitamin A on manganese dioxide (*cf.* Wald, 1947–48; Wald and Brown, 1953–54). The vitamin A was a gift from Dr. N. Embree of Distillation Products Industries in Rochester, New York. Neo-*b* and neo-*a* retinene were prepared by Mr. P. K. Brown from the all-trans isomer (*cf.* Brown and Wald, 1956). Iso-*a* retinene was a gift from Dr. Baxter of Distillation Products Industries, and was prepared by the method of Robeson *et al.* (1955). To dissolve retinene in aqueous digitonin, a drop of retinene concentrate in ethyl alcohol is added to an appropriate volume of 1 per cent digitonin. A voluminous precipitate usually forms, most of which disappears after an hour at 4°C. The solution can then be clarified further by drawing it through a sintered glass filter. This procedure yields aqueous solutions with extinctions of 30 to 40 (containing about 1 micromole retinene per ml.).

Neo-*b* vitamin A was prepared by reducing crystalline neo-*b* retinene with potassium borohydride (KBH_4) (Brown and Wald, 1956). The vitamins A (all-trans and neo-*b*) were brought into aqueous solution by mixing chloroform solutions of vitamin A and tween 80, removing chloroform under suction, and dissolving the residue in distilled water (*cf.* Bliss, 1951 *b*).

Assays for Neo-b and Iso-a Retinene.—The condensation of neo-*b* retinene with opsin provides a sensitive assay method (*cf.* Hubbard, Gregerman, and Wald, 1952–53). Excess opsin is added to a retinene sample and the mixture incubated in the dark for about 2 hours. Hydroxylamine is then added at a final concentration of 0.15 to 0.2 M, and the absorption spectrum measured before and after bleaching. The change in extinction at 500 mμ yields a measure of the amount of rhodopsin. Rhodopsin has a molar extinction (E(1 M, 1 cm., 500 mμ)) of 40,600 (Wald and Brown, 1953–54), and contains one molecule of retinene (Hubbard, 1953–54). The neo-*b* content of a solution is therefore equivalent on a molar basis to the amount of rhodopsin formed on incubation with excess opsin.

When iso-*a* retinene is also present, the neo-*b* assay is complicated by the fact that iso-*a* condenses with opsin, forming a second photosensitive pigment, isorhodopsin (Hubbard and Wald, 1952–53). Isorhodopsin has its absorption maximum at 486 mμ and a molar extinction (E (1 M, 1 cm., 486 mμ)) of 43,000.[10]

The composition of a mixture of isorhodopsin and rhodopsin can be analyzed from the shape of the difference spectrum. With pure rhodopsin, the ratio of extinctions at the absorption maximum and at four other wave lengths (540, 530, 470, and 460 mμ) is as follows:—

$$\frac{K_{540}}{K_{500}} = 0.585, \quad \frac{K_{530}}{K_{500}} = 0.756, \quad \frac{K_{470}}{K_{500}} = 0.798, \quad \text{and} \quad \frac{K_{460}}{K_{500}} = 0.675.$$

The corresponding ratios for isorhodopsin are:

$$\frac{K_{540}}{K_{486}} = 0.335, \quad \frac{K_{530}}{K_{486}} = 0.493, \quad \frac{K_{470}}{K_{486}} = 0.921, \quad \text{and} \quad \frac{K_{460}}{K_{486}} = 0.815.$$

From these one can derive two sets of equations, each of which yields the extinction of isorhodopsin (K_{486}) or rhodopsin (K_{500}) as a function of the extinctions of the mixture at a pair of wave lengths: for instance, 540 and 460 mμ, or 530 and 470 mμ. Thus for isorhodopsin,

$$K_{486} = 2.32\,K_{460} - 2.68\,K_{540}, \text{ or alternatively,}$$
$$K_{486} = 2.51\,K_{470} - 2.65\,K_{530}.$$

And for rhodopsin,

$$K_{500} = 3.24\,K_{540} - 1.33\,K_{460}, \text{ or}$$
$$K_{500} = 3.05\,K_{530} - 1.63\,K_{470}.$$

The two sets of values usually agree within a few per cent and are averaged. In the presence of a fiftyfold excess of either isomer, the results are accurate to within about 5 per cent.

Alcohol dehydrogenase was prepared by extracting retinas with buffer and collecting the fraction which precipitates between 50 and 80 per cent saturation with ammonium sulfate. This was dissolved in the minimum amount of buffer and dialyzed.

[10] The extinction of isorhodopsin is 6 per cent higher than that of rhodopsin (Hubbard and Wald, 1952–53). Since rhodopsin has a molar extinction (E (1 M, 1 cm., 500 mμ)) of 40,600 (Wald and Brown, 1953–54), the corresponding value for isorhodopsin (E (1 M, 1 cm., 486 mμ)) is 43,000.

If the tissues are fresh, the extract contains no hemoglobin, a great advantage for spectrophotometry over similarly crude preparations from liver.

DPN was obtained from Schwarz Laboratories, and ***digitonin*** from Hoffmann-La Roche, Inc. Solutions of ***hydroxylamine*** (1 M) were prepared by dissolving the hydrochloride in distilled water and neutralizing to pH 6.3 with sodium hydroxide.

All solutions were prepared in glass-distilled water.

SUMMARY

Rhodopsin is formed by the condensation of opsin with a cis isomer of retinene, called neo-*b*. The bleaching of rhodopsin releases all-trans retinene which must be isomerized back to neo-*b* in order for rhodopsin to regenerate. Both retinene isomers are in equilibrium with the corresponding isomers of vitamin A, through the alcohol dehydrogenase system.

An enzyme is found in cattle retinas and frog pigment layers which catalyzes the interconversion of all-trans and neo-*b* retinene. We call it "retinene isomerase." It is soluble in neutral phosphate buffer, and precipitates between 20 and 35 per cent saturation with ammonium sulfate.

In the dark, the isomerase converts all-trans and neo-*b* retinene to an equilibrium mixture of 5 parts neo-*b* and 95 parts all-trans. With opsin present to trap neo-*b*, the isomerase catalyzes the synthesis of rhodopsin from all-trans retinene. This reaction, however, is too slow to account for dark adaptation.

Retinene is isomerized by light, but too slowly to supply the retina with neo-*b*. In aqueous solution the pseudoequilibrium mixture contains about 15 per cent neo-*b*.

When all-trans retinene is irradiated *in the presence of isomerase*, the rate of formation of neo-*b* is increased about 5 times, and the pseudoequilibrium shifted so that the mixture now contains about 32 per cent neo-*b*.

The isomerase is specific for all-trans and neo-*b* retinene. It does not act on two other cis isomers of retinene, nor on all-trans or neo-*b* vitamin A.

The role of the isomerase in vision appears to be as follows: in the light, as rhodopsin is bleached to opsin and all-trans retinene, the latter is in part converted to the neo-*b* isomer and stored in the pigment epithelium as neo-*b* vitamin A. During dark adaptation, the dominant process is the trapping by opsin of neo-*b* retinene supplied from stores of neo-*b* vitamin A, and the slow isomerase-catalyzed "dark" conversion of all-trans to neo-*b* retinene.

REFERENCES

Ames, S. R., Swanson, W. J., and Harris, P. L., Biochemical Studies on vitamin A. XV. Biopotencies of geometric isomers of vitamin A aldehyde in the rat, *J. Am. Chem. Soc.*, 1955, **77**, 4136.

Bliss, A. F., Properties of the pigment layer factor in the regeneration of rhodopsin, *J. Biol. Chem.*, 1951 *a*, **193**, 525.

Bliss, A. F., The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase, *Arch. Biochem. and Biophysic.*, 1951 *b*, **31**, 197.

Brown, P. K., and Wald, G., The neo-*b* isomer of vitamin A and retinene, *J. Biol. Chem.*, 1956, in press.

Campbell, F. W., and Rushton, W. A. H., Measurement of the scotopic pigment in the living human eye, *J. Physiol.*, 1955, **130,** 131.

Collins, F. D., Green, J. N., and Morton, R. A., Studies in rhodopsin. 6. Regeneration of rhodopsin, *Biochem. J.*, 1953, **53,** 152.

Collins, F. D., Green, J. N., and Morton, R. A., Studies in rhodopsin. 7. Regeneration of rhodopsin by comminuted ox retina, *Biochem. J.*, 1954, **56,** 493.

Granit, R., Therman, P. O., and Wrede, C. M., Selective effects of different adapting wave-lengths on the dark adapted frog's retina, *Skand. Arch. Physiol.*, 1938, **80,** 142.

Hubbard, R., The molecular weight of rhodopsin and the nature of the rhodopsin-digitonin complex, *J. Gen. Physiol.*, 1953–54, **37,** 381.

Hubbard, R., Retinene isomerase, *Fed. Proc.*, 1955, **14,** 229.

Hubbard, R., Stereoisomerization of vitamin A, retinene, and retinene oxime, *J. Am. Chem. Soc.*, 1956, in press.

Hubbard, R., Gregerman, R. I., and Wald, G., Geometrical isomers of retinene, *J. Gen. Physiol.*, 1952–53, **36,** 415.

Hubbard, R., and Wald, G., The mechanism of rhodopsin synthesis, *Proc. Nat. Acad. Sc.*, 1951, **37,** 69.

Hubbard, R., and Wald, G., Cis-trans isomers of vitamin A and retinene in the rhodopsin system, *J. Gen. Physiol.*, 1952–53, **36,** 269.

Milkman, R. D., and Kennedy, D., Modification of spectral sensitivity by the lens, *Am. J. Physiol.*, 1955, **183,** 645.

Oroshnik, W., The synthesis and configuration of neo-b vitamin A and neoretinene b, *J. Am. Chem. Soc.*, in press.

Pauling, L., Recent work on the configuration and electronic structure of molecules; with some applications to natural products, *Fortschr. Chem. org. Naturstoffe*, 1939, **3,** 203.

Pauling, L., Zur *cis-trans*-Isomerisierung von Carotinoiden, *Helv. Chim. Acta*, 1949, **32,** 2241.

Robeson, C. D., and Baxter, J. G., Neovitamin A, *J. Am. Chem. Soc.*, 1947, **69,** 136.

Robeson, C. D., Blum, W. P., Dieterle, J. M., Cawley, J. D., and Baxter, J. G., Chemistry of vitamin A. XXV. Geometrical isomers of vitamin A aldehyde and an isomer of its alpha-ionone analog, *J. Am. Chem. Soc.*, 1955, **77,** 4120.

Rushton, W. A. H., Campbell, F. W., Hagins, W. A., and Brindley, G. S., The bleaching and regeneration of rhodopsin in the living eye of the albino rabbit and of man, *Optica Acta*, 1955, **4,** 183.

St. George, R. C. C., The interplay of light and heat in bleaching rhodopsin, *J. Gen. Physiol.*, 1951–52, **35,** 495.

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

Wald, G., Human vision and the spectrum, *Science*, 1945, **101,** 653.

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

Wald, G., The photochemistry of vision, *Documenta Ophthalmologica*, 1949, **3,** 94.

Wald, G., and Brown, P. K., The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin, *J. Gen. Physiol.*, 1951–52, **35,** 797.

Wald, G., and Brown, P. K., The molar extinction of rhodopsin, *J. Gen. Physiol.*, 1953–54, **37,** 189.

Wald, G., and Brown, P. K., The synthesis and bleaching of rhodopsin, *Nature,* 1956, **175,** 390.

Wald, G., Brown, P. K., Hubbard, R., and Oroshnik, W., Hindered *cis* isomers of vitamin A and retinene: the structure of the neo-*b* isomer, *Proc. Nat. Acad. Sc.*, 1955, **41,** 438.

Wald, G., and Burg, S. P., Crustacean vitamin A, *Fed. Proc.*, 1955, **14,** 300.

Wald, G., Durell, J., and St. George, R. C. C., The light reaction in the bleaching of rhodopsin, *Science*, 1950, **111,** 179.

Wald, G., and Hubbard, R., The synthesis of rhodopsin from vitamin A_1, *Proc. Nat. Acad. Sc.*, 1950, **36,** 92.

Zechmeister, L., Cis-trans isomerization and stereochemistry of carotenoids and diphenylpolyenes, *Chem. Rev.*, 1944, **34,** 267.

Zewi, M., On the regeneration of visual purple, *Acta Soc. Scient. Fenn.*, *Series B*, 1939, **2,** No. 4.