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## Synthesis of Rhodopsin and Opsin in Vitro<sup>†</sup>

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**ABSTRACT:** Isolated bovine retinas have been used to study the synthesis of rhodopsin and the renewal of photoreceptor rod outer segments. Both leucine and glucosamine served as radioactive tracers to follow rhodopsin synthesis. In both cases the rod outer segment preparations contained large amounts of labeled macromolecules chromatographically distinct from rhodopsin, the latter representing only about 10% of the high molecular weight labeled material. However, electrophoresis on polyacrylamide gels with sodium dodecyl sulfate indicated that as much as 60-68% of the radioactivity coincided with opsin, the apoprotein of rhodopsin. Treatment of labeled rod outer segments with 9-*cis*-retinal caused much of the non-rhodopsin label to be converted

to isorhodopsin. After such treatment the fraction of the label in visual pigment rose from about 10 to 51% with leucine as the radioactive tracer and to 78% with glucosamine. Similar treatment of bleached outer segments labeled with leucine gave identical results with complete regeneration of isorhodopsin ( $\lambda_{\max}$  487 nm) which then accounted for 56% of the labeled macromolecules. No such conversion occurred in controls lacking 9-*cis*-retinal. Both 9-*cis*- and 11-*cis*-retinal were effective but *all-trans*-retinal was ineffective in producing the conversion. Under in vitro conditions opsin appears to be accumulated in the outer segment prior to the addition of retinal.

**B**iochemical studies on the biosynthesis of rhodopsin were initially carried out in whole animals (Hall et al., 1968, 1969; Matsubara et al., 1968; Bargoot et al., 1969).

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Subsequent work in vitro has involved the use of isolated retinas (O'Brien et al., 1972; Basinger and Hall, 1973; O'Brien and Muellenberg, 1973, 1974; Bok et al., 1974). In working with bovine retina, we have consistently found in outer segment preparations highly labeled opsin-like proteins which we have recently succeeded in converting to visual pigment (O'Brien and Muellenberg, 1974). However, no such striking accumulations of opsin are observed in the

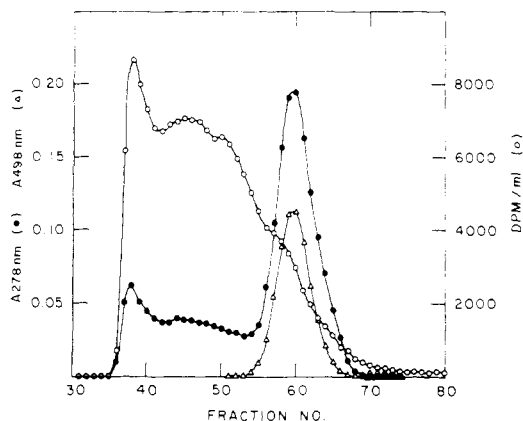


FIGURE 1: Agarose chromatography of [ $^3\text{H}$ ]leucine labeled rod outer segment extract. Extraction and chromatography both carried out with a solution of 1% Emulphogene BC 720 in 0.05 *N* Tris-HCl (pH 8.5). Extract was applied directly to a column of Bio-Gel A 1.5 m, 100–200 mesh. The flow rate was 12 ml/hr and 3-ml fractions were collected, of which 1 ml was taken for scintillation counting. All operations were performed in the dark or under dim red light. Absorbances at two wavelengths are plotted along with disintegrations per min per ml for each fraction.

outer segments of frog retinas in vitro.

In earlier work with labeled leucine and glucosamine it was noted that large quantities of radioactive non-rhodopsin macromolecules could be removed from outer segment extracts by chromatographic methods (O'Brien, et al., 1972; O'Brien and Muellenberg, 1973). It was not known whether this radioactivity represented contamination of the outer segment preparation with other cell organelles or authentic outer segment proteins. We wish to present evidence that these macromolecules are not contaminants and that they are, in fact, primarily opsin.

## Methods

**Preparation and Incubation of Retinas.** Bovine eyes were obtained fresh from a slaughterhouse and the retinas were removed and incubated as previously described (O'Brien and Muellenberg, 1974). Retinol palmitate was obtained from Sigma, St. Louis, Mo.

**Preparation and Extraction of Rod Outer Segments.** The isolation of highly purified rod outer segments was accomplished by a stepwise sucrose gradient method (Papermaster and Dreyer, 1974) described in detail elsewhere (O'Brien and Muellenberg, 1974) as is the extraction of outer segments with Emulphogene BC-720 (General Aniline and Film Corp., New York, N.Y.). Extracts were chromatographed in the dark on agarose (O'Brien et al., 1972) either directly or after a prior passage over calcium phosphate-Celite (O'Brien and Muellenberg, 1974). Spectrophotometric and radioactivity measurements have also been described previously (O'Brien and Muellenberg, 1974).

**Incubation of Rod Outer Segments with 9-*cis*-Retinal.** Rod outer segments from four retinas were suspended in 3 ml of 66 mM sodium phosphate buffer (pH 7.1) and treated with 9-*cis*-retinal (Eastman Organic Chemicals, Rochester, N.Y.) as previously described (O'Brien and Muellenberg, 1974).

**Preparation of 11-*cis*-Retinal and all-*trans*-Retinal.** A solution of all-*trans*-retinal (Eastman), 6.5  $\mu\text{mol}$  in 0.4 ml of ethanol, was irradiated at 4° for 3.5 hr under 70 ft-c of fluorescent light. The solution was applied as a 16-cm band to a silica gel thin-layer plate which was developed in the

dark for 1.5 hr with cyclohexane-ether (17:3 v/v) at 4°. Retinal isomers were detected by examination of the edges of the plate with ultraviolet light and the corresponding bands were scraped off and extracted with ethanol. Spectral analysis revealed absorption maxima of 380 nm for 11-*cis*-retinal and 383 nm for all-*trans*-retinal. Both isomers were incubated with rod outer segments as described for 9-*cis*-retinal.

**Electrophoresis of Rod Outer Segment Proteins.** Rod outer segments from four retinas were suspended in 2.5 ml of a solution containing 2.5% (w/v) sodium dodecyl sulfate (SDS),<sup>1</sup> 5% (v/v) 2-mercaptoethanol, and 0.01 *M* sodium phosphate (pH 7.1) (Laico et al., 1970). The suspensions were completely dissolved by heating for 3 hr at 37° and excess detergent was removed by dialysis for 3.5 days at room temperature against two changes of 2 l. of 0.01 *M* sodium phosphate (pH 7.1) containing 0.1% SDS and 0.1% 2-mercaptoethanol. Electrophoresis of 50- $\mu\text{l}$  samples was carried out for 30 min at 40 mA followed by 2.5 hr at 115 mA in a vertical gel electrophoresis cell (E-C Apparatus Corp. St. Petersburg, Fla.) using a 7.5% polyacrylamide gel made up in 0.01 *M* sodium phosphate (pH 7.1) with 0.1% SDS. The electrode buffer was the same (Shapiro et al., 1967). Gels were stained with 0.2% Amido Black in methanol-water-acetic acid (5:5:1; v/v) and destained with the same solvent. Gels were cut into 2-mm slices except in the region of high radioactivity where 1-mm slices were taken. Gel slices were incubated for 2 hr at 55° in 1 ml of NCS (Amersham/Searle, Arlington Heights, Ill.) with 0.1 ml of water added. After cooling, 10 ml of a toluene-base scintillation fluid was added for radioactivity measurements. Recoveries ranged from 73 to 87% of the radioactivity applied to the gels.

## Results

**Chromatography and Electrophoresis of Rod Outer Segment Extracts.** Rod outer segments were prepared from retinas incubated for 4 hr in the presence of [ $^3\text{H}$ ]leucine, 50  $\mu\text{Ci}/\text{flask}$ , each of which contained two retinas in 10 ml of buffer. The outer segments from 20 retinas were pooled, then divided into five approximately equal portions for various experiments described below. The outer segments from one portion were extracted with Emulphogene and half of the extract was chromatographed on an agarose column to produce the elution profile seen in Figure 1. Most of the radioactivity did not coincide with rhodopsin. As reported previously (O'Brien et al., 1972) this non-rhodopsin labeled protein was largely removed by a prior purification of the other half of the extract on calcium phosphate-Celite. Only a relatively small amount remained as a peak which coincided with bleached rhodopsin (opsin) and was eluted about 10 column fractions ahead of labeled native rhodopsin on agarose. None of the radioactivity seen at the void volume in Figure 1 survived the calcium phosphate-Celite purification step, nor did the 278-nm absorbing material which was eluted prior to rhodopsin on agarose.

In order to determine what portion, if any, of the non-rhodopsin material was related to opsin, another portion of the radioactive outer segment preparation was extracted with SDS and subjected to polyacrylamide gel electrophoresis as described under Methods. The results are shown in Figure 2. Whereas only about 10% of the radioactivity had coincided with rhodopsin on the agarose column, from 60 to

<sup>1</sup> Abbreviations used are: SDS, sodium dodecyl sulfate.

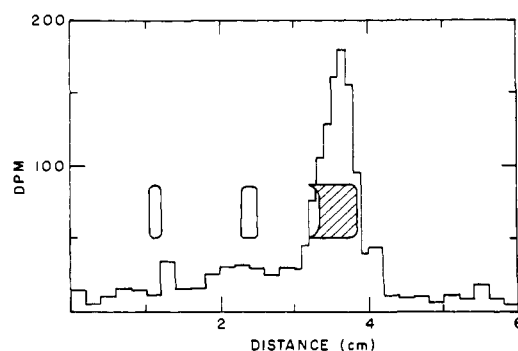


FIGURE 2: Polyacrylamide gel electrophoresis of [ $^3\text{H}$ ]leucine labeled rod outer segment extract in the presence of sodium dodecyl sulfate. Extraction and electrophoresis were as described under Methods. Gel was stained with Amido Black with the major protein band, opsin, shown as the cross-hatched zone. After staining, slices were made and processed for counting as described under Methods. Slices were 2-mm thick in all areas except the opsin zone where 1-mm slices were made. Total disintegrations/min are plotted for each slice.

68% of the radioactivity coincided with opsin on the SDS gels. Rhodopsin was bleached by SDS so that only opsin was present as the major protein band on the gels. Two minor bands were also visible. When Coomassie Blue was used to stain the gels, up to 20 bands of protein in addition to opsin could be visualized, all representing very small fractions of the total protein, as reported by Papermaster and Dreyer (1974). However, no heavy concentrations of radioactivity appeared on the gel in any location other than that of opsin. Thus, it appeared that a large fraction of the radioactive protein in the outer segment extract resembled opsin at least on the basis of molecular weight as determined by SDS-polyacrylamide gel electrophoresis.

**Effect of 9-*cis*-Retinal on Rod Outer Segment Proteins.** In a previous publication (O'Brien and Muellenberg, 1974) it was shown that the small amount of opsin-like radioactive protein, which can be recovered with rhodopsin after passage through a calcium phosphate-Celite column, was converted to visual pigment by treatment of rod outer segments with 9-*cis*-retinal. It was therefore of interest to determine whether the bulk of the outer segment radioactive protein could likewise be converted to visual pigment in view of the apparent relationship to opsin suggested by the SDS gel electrophoresis. Accordingly a portion of the pooled rod outer segments labeled with [ $^3\text{H}$ ]leucine as described above was incubated with 9-*cis*-retinal. The visual pigment was extracted and half of the extract was chromatographed on agarose to produce the elution profile seen in Figure 3. It can be seen that a large fraction of the total radioactivity shifted from the higher molecular weight regions of the elution pattern into the visual pigment peak. Comparison with the control extract (Figure 1) shows that the fraction of the radioactivity associated with the visual pigment peak rose from 10 to 51%. The specific activity of the visual pigment likewise increased from 25,800 to 96,200 dpm per ml per  $A_{498\text{nm}}$ . Specific activity is calculated at 498 nm, the  $\lambda_{\text{max}}$  of rhodopsin, since only a small fraction of the visual pigment is isorhodopsin. No selective losses of either radioactivity or protein occurred since the summation of radioactivity and 278-nm absorbance across the two elution profiles gave ratios of 78,700 dpm/ $A_{278\text{nm}}$  for Figure 1 and 72,300 dpm/ $A_{278\text{nm}}$  for Figure 3. When the other half of the 9-*cis*-retinal treated extract was chromatographed first on calcium phosphate-Celite, then on agarose, only the high molec-

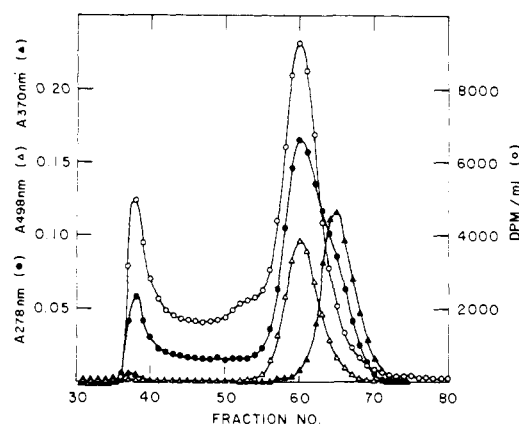


FIGURE 3: Agarose chromatography of an extract of [ $^3\text{H}$ ]leucine labeled rod outer segments which had been incubated with 9-*cis*-retinal. Chromatography was as described for Figure 1. Absorbances of proteins at 278 and 498 nm and of 9-*cis*-retinal at 370 nm are plotted along with disintegrations per min per ml.

ular weight proteins were removed as shown previously (O'Brien and Muellenberg, 1974), leaving a single rhodopsin peak with a specific activity of 88,600 dpm per ml per  $A_{498\text{nm}}$ . Consequently, the calcium phosphate-Celite was no longer able to retain the radioactive protein after its conversion to visual pigment.

It appears that most of the radioactive protein eluting between fractions 40 and 55 was converted to visual pigment by the action of 9-*cis*-retinal. A small amount of 278 nm absorbing material in the same region also appears to have been so converted. This is the region where opsin is known to be eluted (O'Brien et al., 1972). It thus appears that a process similar to regeneration of rhodopsin took place during the incubation of outer segments with 9-*cis*-retinal. In a control experiment another portion of the same [ $^3\text{H}$ ]leucine labeled rod outer segments was incubated in phosphate buffer at the same time as the one shown in Figure 3, but without any addition of 9-*cis*-retinal. The extract was chromatographed on agarose. No conversion of radioactive protein to visual pigment took place, with the specific activity of 29,700 dpm per ml per  $A_{498\text{nm}}$  remaining near the original value. Thus it is apparent that 9-*cis*-retinal was essential for the conversion of the radioactive protein to visual pigment. It is interesting to note that incubation and extraction in phosphate buffer, pH 7.1, caused opsin to aggregate extensively so that it was eluted in the void volume. In contrast, extraction in Tris buffer (pH 8.5) produced lower oligomers, perhaps even monomers, of opsin which were eluted in the zone between fractions 40 and 55 (Figure 1).

**Regeneration of Visual Pigment.** Since the conversion of radioactive protein to visual pigment apparently involved a process similar or identical with regeneration, it became imperative to demonstrate that visual pigment regeneration was possible under the conditions employed for these experiments. To show this, another portion of [ $^3\text{H}$ ]leucine labeled rod outer segments was suspended in 3 ml of 66 mM sodium phosphate (pH 7.1) and exposed to approximately 70 ft-c of white light at room temperature for 1 hr. The sample was divided into two parts and placed in the dark. One part was extracted directly by the addition of Emulphogene and processed as usual. To the other half of the suspension was added 9-*cis*-retinal solution. After a 2-hr incubation at room temperature, Emulphogene was added and extraction was carried out as usual. Both extracts were

Table I: Summary of Operations Performed on Leucine Labeled ROS.<sup>a</sup>

Treatment of ROS	Separation Methods	Major Labeled Protein
None	Electrophoresis, agarose, or CPC-A <sup>b</sup>	Opsin (adsorbed by CPC) <sup>c</sup>
Incubated with 9- <i>cis</i> -retinal <sup>d</sup>	Agarose or CPC-A	Visual Pigment
Incubated without 9- <i>cis</i> -retinal	Agarose	Opsin
Bleached and incubated without 9- <i>cis</i> -retinal	Agarose	Opsin
Bleached and incubated with 9- <i>cis</i> -retinal	Agarose	Visual Pigment

<sup>a</sup>ROS, rod outer segments. <sup>b</sup>Calcium phosphate–Celite followed by agarose. <sup>c</sup>Small but variable amounts of opsin pass through the CPC column. <sup>d</sup>Incubation in 66 mM phosphate (pH 7.1).

chromatographed on agarose. Essentially all the rhodopsin in the first sample was bleached to opsin now aggregated and found in the void volume. In addition, virtually all of the radioactivity was associated with opsin. The bleached visual pigment in the sample treated with 9-*cis*-retinal was regenerated to give an elution pattern virtually identical with that seen in Figure 3 where outer segments had been exposed to 9-*cis*-retinal without prior bleaching. Fifty-six percent of the total radioactivity in the sample was associated with the regenerated visual pigment, isorhodopsin, having an absorption maximum at 487 nm and a spectrum identical with that shown by Shichi and Somers (1974). The specific activity of the isorhodopsin formed was 89,700 dpm per ml per  $A_{487\text{nm}}$ . Thus visual pigment regeneration actually occurred under these conditions and the shift observed in the elution pattern of the radioactive protein in both this and the earlier experiment was probably caused by a process resembling regeneration. The preceding experiments and controls are summarized in Table I.

**Conversion of Glucosamine Labeled Opsin to Visual Pigment.** As reported previously (O'Brien and Muellenberg, 1973) a large amount of non-rhodopsin labeled material can also be found in extracts of rod outer segments after incubation with [<sup>3</sup>H]glucosamine. Experiments similar to those just described were carried out using outer segments prepared from 16 retinas incubated for 4 hr in the presence of [<sup>3</sup>H]glucosamine, 50  $\mu\text{Ci}/\text{flask}$ , each flask containing two retinas in 10 ml of buffer. One portion of the pooled outer segment preparation representing four retinas was extracted with Emulphogene and half of the extract was chromatographed on an agarose column. A large portion of the radioactive material was eluted prior to rhodopsin with only 11% coinciding with rhodopsin, similar to the distribution shown in Figure 1 with leucine as the radioactive label. A noticeable peak occurred in the region of the elution profile where opsin was known to be eluted with somewhat less radioactivity at the void volume. As reported previously (O'Brien and Muellenberg, 1974) prior treatment of the other half of the extract by passage over a calcium phosphate–Celite column removed a large portion of the radioactive material, leaving roughly equivalent peaks coinciding with opsin and rhodopsin, a pattern virtually identical with that seen with leucine labeled outer segment extracts similarly treated.

Another portion of the pooled outer segment preparation

Table II: Specificity of Retinal Isomer.

Treatment of Rod Outer Segments	Specific Activity of Rhodopsin (dpm per ml per $A_{498\text{nm}}$ )
Unincubated control	35,451
Incubated with 0.28 $\mu\text{mol}$ of 9- <i>cis</i> -retinal <sup>a</sup>	179,097
Incubated with 0.23 $\mu\text{mol}$ of 11- <i>cis</i> -retinal <sup>a</sup>	161,977
Incubated with 0.26 $\mu\text{mol}$ of <i>all-trans</i> -retinal <sup>a</sup>	39,697
Incubated with 50 $\mu\text{l}$ of ethanol	49,604

<sup>a</sup>Added in 50  $\mu\text{l}$  of ethanol.

was treated with 9-*cis*-retinal as described above and extracted with Emulphogene. The radioactivity was largely converted to visual pigment with 78% associated with rhodopsin, as in Figure 3, compared with 11% in the control. The specific activity of the visual pigment increased from 25,200 to 102,700 dpm per ml per  $A_{498\text{nm}}$ . As in the previous experiments with leucine the summed radioactivity and 278 absorbance gave ratios of 56,600 dpm/ $A_{278\text{nm}}$  in the control and 55,700 dpm/ $A_{278\text{nm}}$  in the sample treated with 9-*cis*-retinal. Once again, no selective losses of either protein or radioactivity occurred during any manipulations. Prior chromatography on calcium phosphate–Celite, as before, produced a single visual pigment peak on agarose, having a specific activity of 111,300 dpm per ml per  $A_{498\text{nm}}$ . Thus the treatment of outer segments with 9-*cis*-retinal caused a conversion of most of the radioactive material from a species resembling opsin and almost totally adsorbed by calcium phosphate–Celite, to a species apparently identical with visual pigment and no longer adsorbed by calcium phosphate–Celite. No such conversion occurred if 9-*cis*-retinal was omitted from the incubation of the outer segments.

**Specificity of Retinal Isomer.** The naturally occurring isomer of retinal which serves as the chromophore of rhodopsin is 11-*cis*-retinal, not 9-*cis*-retinal which was used in the preceding experiments because of stability and ease of handling. Consequently, the following experiment was carried out to demonstrate the validity of using 9-*cis*-retinal routinely and to eliminate the possibility that nonspecific binding of any retinal isomer could produce the results observed.

Both 11-*cis*- and *all-trans*-retinal were purified as described under Methods and tested along with 9-*cis*-retinal using [<sup>3</sup>H]leucine labeled rod outer segments, prepared as before. Table II shows that incubation of rod outer segments with either 9-*cis*- or 11-*cis*-retinal caused the conversion of radioactive protein to visual pigment, as indicated by the increase in specific activity, producing elution profiles identical with those in Figure 3. However, neither *all-trans*-retinal nor ethanol, used as a solvent for the addition of the retinal isomers, had any effect. The results are therefore specifically dependent on the natural isomer, 11-*cis*-retinal, or one sufficiently similar in structure to serve as a chromophore for visual pigment regeneration such as 9-*cis*-retinal. As additional controls, retinas were incubated with [<sup>3</sup>H]leucine in the usual way but with the addition of either 0.5  $\mu\text{mol}$  of retinol palmitate or 0.09  $\mu\text{mol}$  of *all-trans*-retinal to each flask. In neither case did the elution profile of the outer segment extracts differ from that of the control lacking the additions showing, once again, the requirement for the *cis* isomers and suggesting that a deficiency of meta-

bolic precursors of the chromophore does not account for the accumulation of opsin. These results are not in agreement with the observations of Amer and Akhtar (1972a,b, 1973) who reported the isomerization of *all-trans*-retinal to 11-*cis*-retinal and regeneration of rhodopsin in crude bovine rod outer segments as well as in isolated rat retinas.

### Discussion

A large fraction of the radioactive material extracted from bovine rod outer segments labeled in vitro with either leucine or glucosamine has been found to have the properties of opsin, rather than rhodopsin. Three criteria have been employed in making this determination: the migration of the radioactivity on SDS gel electrophoresis and on agarose chromatography and the ability to "regenerate" visual pigment from the radioactive material. In previous studies (O'Brien et al., 1972; O'Brien and Muellenberg, 1973, 1974) this material was found to be almost totally adsorbed by calcium phosphate-Celite whereas visual pigment was not adsorbed nor was the radioactive visual pigment produced from opsin by treatment of outer segments with 9-*cis*-retinal. In the present studies it was found that the most abundant single component of the non-rhodopsin labeled protein was opsin and that lesser amounts of radioactive protein were widely distributed both on gel electrophoresis and on agarose chromatography. It is of interest to note that the agarose elution profiles produced after 9-*cis*-retinal treatment of bovine rod outer segments bear a very strong resemblance to those obtained with labeled frog rod outer segment extracts by Basinger and Hall (1973) using [<sup>3</sup>H]leucine, and by Bok et al. (1974) using [<sup>3</sup>H]glucosamine. However, no treatment of frog rod outer segments with retinal was necessary to produce the results. Since the methods of incubation, rod outer segment isolation, and chromatography were similar in all studies, it can be inferred that a species difference is the cause of the observed differences in labeling patterns in the absence of retinal treatment. Furthermore, it appears that the addition of retinal to opsin may be the final step in the assembly of functional photoreceptor disc membranes and that the pool of opsin is large in the bovine rod outer segment but small in that of the frog. A structural correlation may exist in that the base of the rod outer segment consists of folds of the plasma membrane which appear to pinch off to produce isolated discs. In the frog there are very few such folds (Cohen, 1972; Young and Droz, 1968) whereas the rhesus monkey outer segment shows a prominent zone of multiple infoldings (Young, 1971). It is tempting to speculate that the size of the opsin pool might be a reflection of the number of basal folds in the outer segment. A detailed study of the bovine retina by electron microscopy would be necessary to establish such a correlation. No such study is presently available so this suggestion must remain purely speculative.

The evidence presented here supports the thesis that under in vitro conditions newly synthesized opsin is accumulated and transported to the photoreceptor rod outer segment prior to the acquisition of the chromophore, retinal. This is consistent with the observation of Basinger and Hall (1973) that opsin is synthesized and accumulated in the outer segments in vitro at the same rate in darkness and in bleaching light where no chromophore could be present. The location of this pool of opsin, possibly in the basal folds of the outer segment, is a matter currently under investiga-

tion. Contamination of outer segment preparations with opsin-containing membranes from the inner segments is a possibility that cannot be ruled out. However, reisolation of outer segments by the stepwise gradient method does not alter the distribution of radioactivity, opsin and rhodopsin on agarose, even though small amounts of high molecular weight protein can be removed. At present it appears that the pool of opsin is probably contained in the outer segments, but this point awaits rigorous demonstration.

The binding of a chromophore to the newly synthesized opsin appears to be specific in that both 9-*cis*-retinal and 11-*cis*-retinal are bound successfully whereas *all-trans*-retinal is not. This is in keeping with the well-known specificity of opsin for the *cis* isomers of retinal in the process of regeneration of light-sensitive visual pigments following bleaching (Wald, 1953). It remains to be seen if the addition of retinal to newly synthesized opsin during outer segment renewal proceeds by the same mechanism employed by the photoreceptor for regeneration of bleached rhodopsin. The striking differences observed by Hall and Bok (1974) in the labeling of frog rhodopsin in vivo with radioactive vitamin A as a function of light suggest that there may be two distinct mechanisms but conclusive proof is lacking. The failure of *all-trans*-retinal to convert newly synthesized opsin to rhodopsin could also suggest different mechanisms for regeneration and renewal. However, there is no evidence that *all-trans*-retinal was actually isomerized in these experiments to 11-*cis*-retinal as reported by Amer and Akhtar (1972a,b, 1973). If it were isomerized it is possible that it could not gain access to the newly synthesized opsin. But since there was no regeneration of bleached rhodopsin by *all-trans*-retinal comparable to that produced by 9-*cis*-retinal (as evidenced by the decrease in 278-nm absorbance in the opsin region in Figure 3 compared with Figure 1) it seems more likely that no isomerization took place. In fact, these experiments indicate that *all-trans*-retinal may not be isomerized in the dark by either rod outer segments or intact bovine retina. Recent work by Shichi and Somers (1974) points instead to a nonenzymic isomerization of *N*-retinylidenephosphatidylethanolamine requiring light as the energy source.

The formation of labeled rhodopsin during the incubation of bovine retinas is progressive with time (O'Brien and Muellenberg, 1974) and suggests the existence of a pool of some form of chromophore available for new disc synthesis and not exhausted during the course of the incubation. Opsin, present in the outer segments as a result of the inability to dark-adapt slaughterhouse animals, persists during the incubations (Figure 1), yet some newly synthesized opsin molecules receive a chromophore. These observations suggest that the addition of chromophore under these experimental conditions may be rate-limiting and possibly restricted to newly synthesized opsin molecules.

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## Interaction of Effecting Ligands with *Lac* Repressor and Repressor–Operator Complex<sup>†</sup>

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**ABSTRACT:** The equilibrium association constants for the binding of a wide variety of effecting ligands of the *lac* repressor were measured by equilibrium dialysis. Also, detailed investigations of the apparent rate of dissociation of repressor–operator complex as a function of ligand concentration were carried out for several inducers and anti-inducers. The affinity of repressor–ligand complex for operator DNA was evaluated from the specific rate constants at saturating concentrations of effecting ligand. By fitting the experimental data depicting the functional dependence of the rate of dissociation upon ligand concentrations to calculated curves, assuming simple models of the induction mecha-

nism, the equilibrium association constant for the binding of effecting ligand to repressor–operator complex was determined. Inducers reduce the affinity of *lac* repressor for operator DNA by a factor of approximately 1000 under standard conditions; the extent of destabilization depends on  $Mg^{2+}$  ion concentration. Anti-inducers increase the affinity of repressor for operator at most a factor of five. Only one neutral ligand, which binds to repressor without altering the stability of repressor–operator complex, was found. No homotropic or heterotropic interactions in the binding of effecting ligands either to repressor or to repressor–operator complex are evident.

In the past 20 years, there have been a number of accounts of the specificity of induction of the lactose operon in *Escherichia coli*. The in vivo studies were primarily concerned with identifying effecting ligands of the *lac* repressor, the majority of which have turned out to be  $\beta$ -galactosides. In the first paper on this subject, Monod et al. (1951) investigated the effect of substitution of several functional groups at different positions on the galactose ring upon induction

of the synthesis of  $\beta$ -galactosidase. Later systematic studies by Müller-Hill et al. (1964) and by Boos et al. (1967) enlarged the list of compounds examined, and classified a number of these sugars as inducers or anti-inducers of the operon. Müller-Hill et al. (1964) also attempted to describe those features of the sugar moiety which are important in making the molecule an effecting ligand, either inducing or anti-inducing. These studies with synthetic ligands yielded much valuable information; more recently, it has been demonstrated that allolactose is the natural inducer of the *lac* operon in vivo (Jobe and Bourgeois, 1972a).

With the isolation of the *lac* repressor protein, it became possible to study induction in vitro. The membrane filtration technique, using purified *lac* repressor and <sup>32</sup>P-labeled phage DNA bearing the *lac* operon, permitted examination of the effects of inducing and anti-inducing ligands upon the equilibrium and kinetic properties of the interaction of repressor protein and operator DNA. In the initial study of a few effecting ligands (Riggs et al., 1970b), the essential features of earlier experiments in vivo were verified. Compounds that were inducers or anti-inducers of  $\beta$ -galactosid-

<sup>†</sup> From the Salk Institute for Biological Studies, San Diego, California 92112, and the City of Hope National Medical Center, Duarte, California 91010. Received October 30, 1974. This research was supported by grants from the National Institutes of Health (GM20259, GM20868, HD04420) and the National Science Foundation (GB 29559 X, GB 26517).

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<sup>§</sup> Recipient of a Career Development Award from the National Institutes of Health.

<sup>#</sup> Supported by a training grant the Institute of Allergy and Infectious Diseases to Dr. Melvin Cohn.