The Gecko Visual Pigments

The Behavior of Opsin

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ABSTRACT The 521-pigment extracted out of the retina of the Tokay gecko has the typical stereospecificity of the vertebrate visual pigments. This is true for the pigment in the chloride-depleted, "blue-shifted" state as well as for the normal pigment with added chloride. While in the chloride-deficient state, pigment regeneration occurred with both 11-*cis*- and 9-*cis*-retinals and the regenerated photopigments were also in the blue-shifted, chloride-depleted state. As with the native pigment, these regenerated pigments were bathochromically shifted to their normal positions by the addition of chloride. Chloride-deficient opsin by itself also responded to chloride for the pigment regenerated with 11-*cis*-retinal from such chloride-treated opsin was in the normal 521-position. Regeneration was always rapid, reaching completion in <5 min, and was significantly faster than for cow rhodopsin regenerating under the same conditions. This rapid rate was found with or without chloride, with both 11-*cis*- and 9-*cis*-retinals and in the presence of the sulfhydryl poison, *p*-hydroxymercuribenzoate (PMB). Like the native chloride-deficient pigment, the regenerated chloride-depleted photopigments responded to PMB by a blue shift beyond the position of the chloride-deficient state. The addition of chloride to these "poisoned" regenerated pigments caused a bathochromic shift of such magnitude as to indicate a repair of both the PMB and chloride-deficient blue shift. In this discussion the possible implications of these results to phylogenetic considerations are considered as well as to some molecular properties of the 521-pigment.

INTRODUCTION

The visual cells of the Tokay gecko (*Gekko gekko*) have two photopigments (P-521 and P-467) which have been detected by spectrophotometric analysis of the pigments *in situ* and in retinal extracts (Crescitelli, 1977 *a*). One of these pigments, P-521, is unusual in a number of respects, when compared with the rhodopsins that have been studied. The location of its maximum at 521 nm, rather than near 500 nm, is notable. So is the reversible spectral change with mild temperature increases as well as the bleaching, in the dark, by hydroxylamine (NH_2OH) and sodium borohydride ($NaBH_4$). Under the same circumstances P-467 shows none of these properties. In addition, P-521, unlike rhodopsin, undergoes a "blue shift" in spectral absorbance when it is treated with the sulfhydryl poison, *p*-hydroxymercuribenzoate (PMB). This apparently

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is an attack by the mercurial on available -SH groups since the blue shift is reversed or prevented by dithiothreitol, the specific thiol-protective reagent of Cleland.

The unusual sensitivity of P-521 to temperature and to NH_2OH relates this gecko pigment more nearly to the cone pigment iodopsin than to any rhodopsin now known (Bliss, 1946; Wald et al., 1955, Crescitelli, 1977 *a*). Even more striking a similarity is the behavior toward chloride ions of both P-521 and chicken iodopsin. This chloride ionochromic effect has been observed to occur with P-521 in extracts prepared with digitonin, with Triton X-100 and with other detergent solubilizers (Crescitelli, 1977 *a*, 1977 *b*) as well as with the P-521 *in situ* within outer segment particles (Crescitelli, 1978). The same responses to chloride have been reported to occur for chicken iodopsin (Knowles, 1976; Fager and Fager, 1977). All these similarities between P-521, a pigment of the so-called gecko rods, and the cone pigment, iodopsin, call to mind the transmutation theory of Walls, 1934), whereby the gecko rods are conceived as visual cells in process of evolutionary transition from ancestral photopic precursors of the diurnal lizard stock that gave rise to geckos. In this connection it needs to be pointed out that the P-521 system of *Gekko gekko* is, in its behavior, typical of gekkonid retinal photopigments. I have examined a number of species and have concluded that the unusual behavior illustrated by P-521 is typical of this lacertilian family.

The chloride effect, the theme around which this investigation is built, is this: when the isolated gecko retina is carefully washed with distilled water and buffer and then extracted into 2% digitonin, the spectral maximum is not at 521 nm, as it is normally within the freshly isolated visual cells, but is in the region of 500–505 nm. The photopigment may be said to be in the chloride-deficient state which I shall designate as $P\text{-}Cl^-$. This is not an irreversibly altered state, for the addition of chloride (or bromide) quickly repairs the deficiency and shifts the spectrum toward longer wavelengths, the magnitude of the shift being related to the chloride concentration. Sufficient chloride restores the normal location at 521 nm but the spectrum cannot be shifted further, even with excessive concentration of chloride. I shall refer to the pigment to which chloride has been added as being in the $P\text{-}Cl^+$ state. This ionochromic response is anion-specific, being given to chloride or bromide but not to several other anions that have been tested (Crescitelli, 1977 *b*). The nature of the cation appears to be irrelevant. I have interpreted this effect as a binding of chloride to a specific site of the opsin, leading to a conformational change in this protein that influences the secondary interaction postulated by Dartnall (1957) and Hubbard (1958) as existing between the opsin and the prosthetic group, an interaction whose specific nature is thought to set the specific color of the chromophores in the various visual pigments. In the light of this hypothesis, the chloride effect has fundamental significance in an eventual understanding of the molecular mechanisms that determine the region of absorbance of a retinal pigment.

It is a well-established fact that the vertebrate visual pigments employ as a prosthetic group a specific stereoisomer of retinal or 3-dehydroretinal. Presum-

ably, this specificity also applies for the gecko visual pigments, but I have not yet established this as a fact, and one purpose of this study is to examine the responses of the 521-opsin to the four retinal isomers: 11-*cis*, 9-*cis*, 13-*cis*, and all-*trans*. This will be done for the pigment in both the P-Cl^+ and P-Cl^- states. The object of this comparison is, of course, to determine whether or not the altered conformational state of the chloride-deficient opsin in any way alters the stereospecificity to the retinal isomers. In addition, a further possible relationship between P-521 and iodopsin will be examined, i.e., the rate of regeneration of photopigment in the reaction of 11-*cis* with the 521-opsin. It is already known (Wald and Brown, 1950; Wald et al., 1955) that chicken iodopsin regenerates at a significantly faster rate than do the rhodopsins. How, then, does P-521 fall into this picture? Does it follow the other properties already mentioned in their similarity to iodopsin, or does it have a rhodopsin-like kinetics? These are the specific questions posed in this investigation.

MATERIALS AND METHODS

Standard methods were employed (Hubbard et al., 1971; Crescitelli, 1963), but this study required some minor modifications adapted to the special conditions of the gecko system. Extracts were prepared using, as solubilizer, 2% digitonin (Merck & Co., Inc., Rahway, N.J.) in Tris-maleate buffer at pH 7.3. For each extract, freshly made before every analysis, a 0.3-ml volume of digitonin was used for each batch of four or five retinas. The retinas, first washed in double-distilled water, were immersed in cold 4% potassium alum for ~2 h, and were then washed twice with the distilled water and once with the buffer. In some cases the alum treatment was omitted, but the results then obtained were in no way different, except for the fact that the extracts were of poorer spectral quality. Temperatures during all procedures were never allowed to exceed 5°C.

The analysis involved two aliquots of the same extract in separate microcells. Depending on the specific experiment, both aliquots were kept in the P-Cl^- state, as prepared, or NaCl was added to one aliquot alone, or to both aliquots. The spectra were checked initially in all analyses, and in no case was the familiar bathochromic shift missing after adding chloride. To prepare the opsin the aliquots in the P-Cl^- or P-Cl^+ state were bleached with light at a wavelength between 600 and 630 nm, the light source being a Bausch & Lomb Inc. (Rochester, N.Y.) monochromator. Bleaching time was adjusted so as to completely remove the 521-pigment while leaving the 467-pigment.

To the opsin so obtained a few drops were added of a freshly prepared solution of the retinal isomer made up in 2% digitonin and at a concentration of 1×10^{-4} M. All the precautions noted by Hubbard et al. (1971) were observed in preparing and handling the isomers. Within 2 min after adding the isomer to the aliquots, spectral readings were begun of the optical density at 700 nm and at the spectral maximum of the regenerated pigment. Readings were continued to completion of regeneration after which the entire spectrum from 360 to 700 nm was read. Because the regenerated pigment was contaminated by excess retinal, a few drops of freshly prepared and neutralized 0.01 M $(NH_2OH)_2H_2SO_4$ were added, and after a wait of some 30 min the spectrum of the regenerated pigment was obtained. In some experiments the NH_2OH was added before adding the isomer, and this caused no complication because the concentration used was too low to cause destruction of the photopigment, which does occur at higher NH_2OH concentrations (Crescitelli, 1963), and because regeneration was so rapid that it was completed before any significant amount of isomer had reacted with the NH_2OH. The results were the same whether NH_2OH was added before or after regeneration. In all

experiments the isomers added were in excess of the opsin and the NH_2OH was in excess of the isomers. All final concentrations of additives were computed from weighings made before and after each addition, a Mettler Instrument Corp. (Hightstown, N.J.) microbalance being used for this purpose.

The all-*trans*-, 13-*cis*-, 9-*cis*-, and 11-*cis*-isomers were either purchased from supply houses or were gifts from Paul K. Brown of Harvard, Dr. W. E. Scott of Hoffmann-LaRoche, Inc., Nutley, N.J., and C. D. Bridges of the Baylor College of Medicine. Before testing each of these isomers on the gecko system I examined their behavior with the well-established bovine rhodopsin. The results were in accord with what is known about these isomers in their reactions with cattle opsin (Hubbard and Wald, 1952).

The addition to each pigment aliquot of various reagents led, of course, to dilution of the photopigment. This was corrected for by use of dilution factors obtained by weighings made before and after each addition. Accordingly, all optical densities are expressed in terms of the pigment density originally in the aliquots.

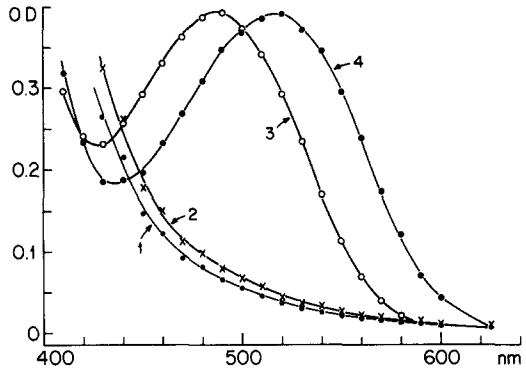

FIGURE 1. The stereospecificity of the gecko system in the P-Cl^+ state. Lack of regeneration with all-*trans*-retinal (curve 1) and with 13-*cis*-retinal (curve 2). Regeneration of isopigment with 9-*cis*-retinal (curve 3) and of the 521-pigment with 11-*cis*-retinal (curve 4). The original spectra of the two aliquots before bleaching are not shown. They were very similar to curve 4. The four aliquots contained NaCl added to give the maximum red shift. The spectral measurements of all four aliquots were made after $(NH_2OH)_2H_2SO_4$ had been added.

RESULTS

Stereospecificity of the P-Cl^+ System

The 521-opsin in the P-Cl^+ state behaved like a typical vertebrate opsin in its responses to the four isomers (Fig. 1). No regeneration was detected with the all-*trans*- (curve 1) or 13-*cis*- (curve 2) retinals, whereas 9-*cis*- (curve 3) and 11-*cis*-retinal (curve 4) led to rapid synthesis of photopigment in considerable yield (60–90%). The 11-*cis*-isomer regenerated the original 521-pigment whereas 9-*cis*-retinal caused the appearance of a new gecko pigment at about 488 nm. Presumably, this is the isopigment comparable to isorhodopsin. It is noteworthy that although the 11-*cis* gecko pigment was shifted bathochromically by some 20 nm, compared to the rhodopsins, the gecko isopigment was at about the same

spectral position as isorhodopsin. In summary, the gecko P-Cl^+ system has the same stereospecificity that characterizes other vertebrate visual pigments.

Stereospecificity of the P-Cl^- System

In the P-Cl^- state the 521-opsin displayed the same stereospecificity as with added chloride. The results, shown in Fig. 2, include only the data for the two active isomers since all-*trans*- and 13-*cis*-retinals yielded no evidence of regeneration. The initial spectra of the extracted pigment in the P-Cl^- state (curves 1A, 2A) show the absorbance maximum to be close to 504 nm for two aliquots of the same extract. After bleaching at 610 nm (curves 1B, 2B) 9-*cis*-retinal was added to aliquot 1, and 11-*cis*-retinal to aliquot 2. Rapid regeneration occurred in both

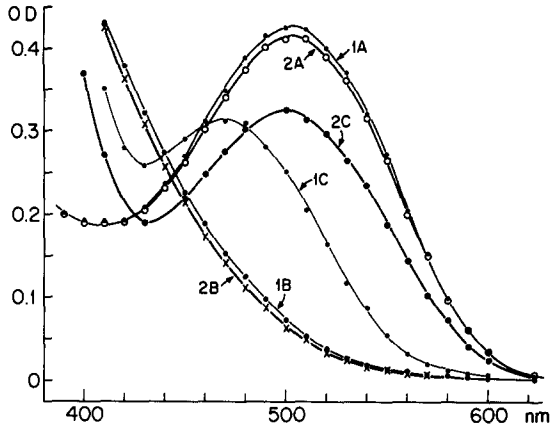

FIGURE 2. The stereospecificity of the gecko system in the P-Cl^- state. The spectra of two aliquots of the same extract in the P-Cl^- state, without added chloride (curves 1A, 2A). After bleaching with light at 610 nm (curves 1B, 2B). The regenerated pigments after 9-*cis*-retinal (curve 1C) and after 11-*cis*-retinal (curve 2C). The readings of curves 1C and 2C were taken after adding $(NH_2OH)_2H_2SO_4$. The results with all-*trans*- and 13-*cis*-retinals are not shown inasmuch as no regeneration occurred to these additions.

aliquots, after completion of which the spectra were read (not shown). Then $(NH_2OH)_2H_2SO_4$ was added to both aliquots, after which the spectra of the regenerated pigments were read (curves 1C, 2C). The 9-*cis*-isomer resulted in a pigment with maximum close to 470 nm, the regeneration being some 63%, whereas 11-*cis*-retinal caused the synthesis of the original 504-pigment to the extent of 75%. These two pigments represent, therefore, the regenerated P-Cl^- pair, counterparts of the regenerated P-Cl^+ pair of Fig. 1. In other words, there is a pair of isopigments, regenerated from 9-*cis*-retinal, one of which is in the P-Cl^- state, the other in the P-Cl^+ state. The pair of pigments regenerated with 11-*cis*-retinal are identical, spectroscopically, to the extracted native pigments, one without, the other with added chloride. All these regenerated pigments are photolabile and bleach in the usual manner upon exposure to light.

Bathochromic Repair of the Regenerated P-Cl⁻ Pigments

The regenerated P-Cl⁻ pigments resulting from the addition of 9-*cis*- and 11-*cis*-retinals to the opsin can, like the native P-Cl⁻, respond to added NaCl by a bathochromic shift. To cite the results of one analysis, the regenerated 11-*cis*-pigment went from 505 nm (P-Cl⁻) to 520 nm (P-Cl⁺) upon the addition of chloride. The 9-*cis*- regenerated pigment shifted from 474 nm (P-Cl⁻) to 486 nm (P-Cl⁺). Accordingly, for both classes (9-*cis* and 11-*cis*) of regenerated photopigments there was a pigment pair separated by a span of 12–15 nm, one member, the chloride-deficient one, located at shorter wavelengths, the second member shifted to longer wavelengths through the action of added chloride.

The Opsin Response to Chloride

There is no question that chloride added to either the native or regenerated pigments in the P-Cl⁻ state induces the bathochromic repair response. It now remains to demonstrate that chloride added to opsin in the P-Cl⁻ state will lead, upon adding the active isomer, to a regenerated pigment that is in the P-Cl⁺

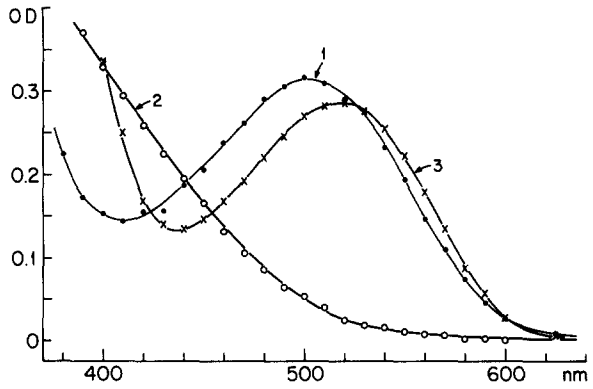

FIGURE 3. The opsin response to chloride. Curve 1: original spectrum of aliquot in the P-Cl⁻ state. Curve 2 (solid line): after bleaching at 600 nm. Curve 2 (○): after adding NaCl to make a concentration of 1.71×10^{-1} M. Curve 3: after 11-*cis*-retinal and measuring spectrum in presence of NH_2OH.

state. Such a result for the case of 11-*cis* retinal is pictured in Fig. 3. The original extracted pigment in the P-Cl⁻ state gave a spectrum with maximum at 502 nm (curve 1). After photic bleaching (curve 2, solid line), NaCl was added to the bleached extract (curve 2, ○). The chloride resulted in no significant change and it is clear that this anion produced no regeneration. The addition of 11-*cis*-retinal led to the typical fast regeneration, which after adding NH_2OH and reading the spectrum, showed the presence of the typical 521-pigment close to 521 nm (curve 3). A comparable result was obtained with the 9-*cis* system. Accordingly, the opsin of itself is capable of responding to chloride by altering its conformation to that of a bathochromically shifted photopigment.

The Kineics of Regeneration

I have already indicated that in all the experiments regeneration was typically rapid, so much so that, without special equipment and techniques, it has not

been possible to establish accurately the velocity of the reaction. By the time the isomer was added, the system mixed, and the cuvette returned to the spectrophotometer compartment, an interval of ~2 min, regeneration was at least 90% completed, and this at a temperature of 5°C. Accordingly, I am presenting a result (Fig. 4), not with the intent of giving the genuine kinetics, but merely to point to the fact that compared to bovine rhodopsin the gecko system regenerates at a significantly greater rate. In this experiment an extract of cattle retinas was compared in its simultaneous behavior with a similarly prepared extract of the gecko pigment placed in a second cuvette. The two pigments were bleached to completion with light at 600 nm after which 11-*cis*-retinal was added, and the rise in optical density at the respective maxima was followed and is shown in Fig. 4 as the percentage of the completed change for the two photopigments. The same rapid regeneration of the gecko pigment was noted after 9-*cis*-retinal, and with or without added chloride for both the 9-*cis* and 11-*cis* systems. The essential point made in this experiment (Fig. 4) is the contrast between the

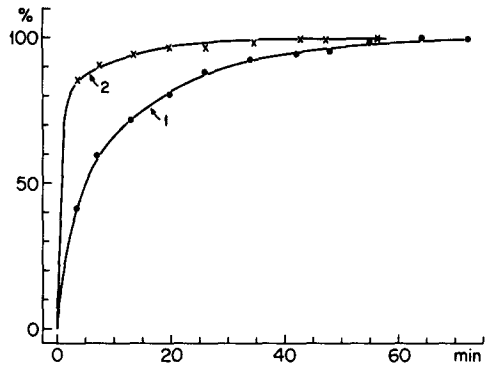

FIGURE 4. Rate of regeneration of bovine rhodopsin (curve 1) and P-Cl^+ gecko pigment (curve 2) after adding 11-*cis*-retinal to bleached extracts. Ordinate: normalized density increments at respective spectral maxima in percentage of completed changes. The two pigments were analyzed simultaneously in two separate microcells.

typical rod pigment, rhodopsin, and the unusual 521-pigment of the gecko. In this respect the gecko pigment is more like iodopsin than rhodopsin.

Along the same lines it is of some interest to inquire into whether or not a sulfhydryl poison blocks the regeneration of the gecko pigment. As noted in the Introduction, *p*-hydroxymercuribenzoate (PMB) was able to attack -SH groups and so to shift the spectrum of the P-Cl^- to shorter wavelengths. This established the fact that this mercurial reagent was able to reach critical sites in the opsin and affect its behavior. Despite this, PMB was found to be unable to block regeneration of the 521-pigment. This result is documented by the experiment of Fig. 5 in which an extract in the P-Cl^- state was divided into aliquots in separate microcells. Aliquot 1 was left as prepared and gave a spectrum with maximum close to 500 nm (curve 1). The mercurial poison was added to aliquot 2 resulting in a spectrum with maximum shifted to ~485 nm (curve 2), a typical PMB effect. The second aliquot was then exhaustively bleached with light at 610

nm, the result being curve 3. The 11-*cis*-isomer was then introduced into the bleached aliquot which led to a rapid regeneration as in Fig. 4. After adding $(NH_2OH)_2H_2SO_4$ to the regenerated pigment, the spectrum was read and is shown as curve 4 (Fig. 5). Sodium chloride was next pipetted in and this led to the "red shift" represented by curve 5. A bleach with light at 620 nm proved that this regenerated pigment was photolabile (curve 6). In another type of experiment in which a pigment in the P-Cl^- state was regenerated by means of 11-*cis*-retinal, the addition of PMB to the regenerated pigment caused a typical blue shift just as in the case of the native P-Cl^-. These results point to the fact that although PMB was able to attack both the native and regenerated systems, it was unable to prevent the regeneration of photopigment or the bathochromic response to chloride.

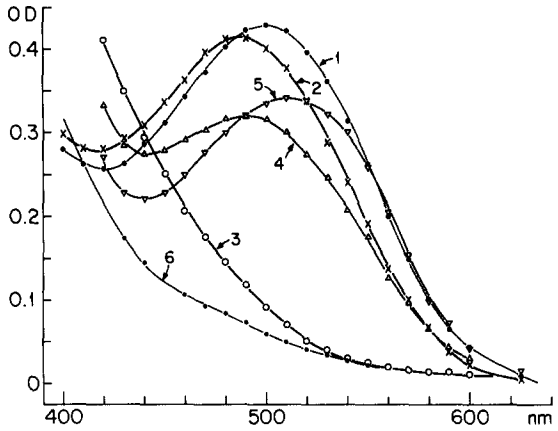

FIGURE 5. Regeneration in presence of *p*-hydroxymercuribenzoate (3.0×10^{-4} M). Curve 1: pigment in original P-Cl^- state. Curve 2: blue shift caused by addition of PMB. Curve 3: after bleaching at 610 nm. Curve 4: pigment regenerated after 11-*cis*-retinal, NH_2OH used. Curve 5: red shift caused by NaCl (9.06×10^{-2} M). Curve 6: final bleach of regenerated pigment with 620 nm.

DISCUSSION

The gecko retina is known to have two photopigments both of which are extractable into digitonin solutions. One of these, absorbing at shorter wavelengths, is present in such extracts as a minor component, quantitatively, and I know little about it except for the fact that its properties seem to be quite different from those of the major component (Crecitelli, 1977 *a*). I now have accumulated enough information on the major component, particularly the 521-pigment of *Gekko gekko,* to make a comprehensive comparison of it with other vertebrate visual pigments, especially the rod pigments known as rhodopsins, on the one hand, and the chicken cone pigment, iodopsin, on the other hand. Such a comparison is of interest in connection with the phylogenetic nature of gecko visual cells which have been considered by Walls (1934) to be in a state of evolutionary intermediacy between photocytes and scotocytes. Walls' hypothesis

has been based solely on morphological criteria and it is abundantly clear that data from other points of view are required to clarify the history of visual cells. Unfortunately, the only cone pigment about which we have much information is iodopsin, so this limits the comparisons that can be made between the gecko main pigments and cone pigments in general.

It has already been established that the gecko main pigments are spectrally located neither in the region of 500 nm, the position of typical rhodopsins, nor at the location of iodopsin, 562 nm, but in a region roughly intermediate between these two. It was this that first called my attention to these pigments. The results of the present study add a piece of information that was missing in my original investigations, i.e., the gecko pigments have the same stereospecificity as do other visual pigments. The opsin reacts with 11-*cis*-retinal, which is the normally occurring prosthetic group, and with 9-*cis*-retinal, but not with the all-*trans*- or the 13-*cis*-isomer. This result was to be expected but it had to be proved, and it now extends the general picture of visual pigment specificity to the reptiles. In the future it will be of some interest to examine the results of reacting the 521-opsin with other isomers of retinal and 3-dehydroretinal and of certain synthetic analogues of retinal, following the lead of Kropf et al. (1973).

With 9-*cis*-retinal the 521-opsin in the P-Cl^+ state regenerates an isopigment located at ~488 nm, close to the position of isorhodopsin. This is of some interest for it indicates that the isoshift is unique for the gecko pigment. By isoshift I mean the blue shift that occurs in going from an opsin linked to 11-*cis*-retinal to the same opsin linked to 9-*cis*-retinal. For bovine rhodopsin the isoshift is in the range of 12–15 nm (Hubbard and Wald, 1952; Wald and Brown, 1956; Yoshizawa and Wald, 1963). It is about the same for frog rhodopsin (Bridges, 1961; Reuter, 1976; Yoshikami and Nöll, 1978) and for elasmobranch rhodopsin (Pepperberg et al., 1978). In contrast, chicken iodopsin has an isoshift of 52 nm (Wald et al., 1955). The present results indicate a value intermediate between these extremes for the gecko 521-opsin, i.e., 31-33 nm. It is also of interest to point out here that in the case of porphyropsin, a rod pigment, the isoshift is of small magnitude, going from 522 to 507 nm upon adding 9-*cis*-3-dehydroretinal to the opsin. Thus, although both the gecko pigment and porphyropsin start in the same position (521 and 522 nm), the former shifts by 32 nm with 9-*cis*-retinal whereas the latter, like the rhodopsins, shifts by only 15 nm. Although more data of this kind are required, especially with cone pigments, the tentative conclusion is that the gecko visual protein has conformational features unlike those of the usual rod pigments and also different from the opsin of iodopsin.

Perhaps the most striking relationship of the gecko pigment system to iodopsin is the specific role of chloride ions in determining the spectral location of both pigments. The fact that both P-521 and iodopsin display this ionochromic response when present *in situ* within the outer segments adds special interest, for it cannot be simply a test tube artifact induced by the detergent. The chloride effect suggests a role for electrostatic forces in adjusting the specific conformation of the opsin associated with the color of the visual pigment. Why this chloride action is not detectable or is absent in rhodopsins is for the moment an unanswerable question. In any case, whatever this specific

conformation is, for the gecko pigment, it is clear that it is not involved in the stereospecific relations of the opsin with the prosthetic group. The P-Cl^- opsin reacts perfectly normally with both 11-*cis*-retinal and with 9-*cis*-retinal, regenerating back the chloride-deficient photopigments which have all the properties of the native pigments, i.e., they respond, for example, with a chloride bathochromic shift. Moreover, the isoshift has the same magnitude for the P-Cl^- system as for the P-Cl^+ counterpart, i.e., 34 nm for the experiment of Fig. 2. In addition to these results is the finding that opsin itself can respond to chloride and alter its conformation so that upon regeneration a red shifted photopigment results (Fig. 3). Chromophoric association between the opsin and retinal is not necessary for the chloride effect.

Not to be neglected is the fact that certain results were not obtained. In the absence of chloride, for example, regeneration might not have occurred or else the stereospecificity might have been altered. Neither of these results was found, supporting the conclusion of the paragraph above that the conformational features associated with chloride are independent of those features that determine the binding of the prosthetic group to the primary site and the specific fit of the retinal within the chromophoric architectural cavity.

A specially convincing link between the 521-pigment and iodopsin is the finding that the regeneration of the 521-system, whatever the conditions, is fast, as it is in the case of iodopsin. Wald et al. (1955) reported the regeneration of iodopsin to be completed within ~3 min at 10°C. This is only slightly faster than the rate for the gecko pigment at 5°C (Fig. 5). In both cases, however, the rate is faster than regeneration of rhodopsins from the cow, chicken, and frog (Wald and Brown, 1950; Wald et al., 1955). Perhaps, associated with this rapid regeneration of the gecko pigment is the very rapid, initial phase of dark adaptation reported by Dodt and Jessen (1961) for certain geckos.

In one respect, however, the gecko pigment is unlike iodopsin as well as rhodopsin, and this is the behavior toward PMB. I have already reported the fact that this mercurial reagent is able to induce a considerable blue shift in the extracted chloride-deficient gecko pigment (Crescitelli, 1975).[1] This is in contrast to the reported absence of effect on the spectrum of rhodopsin (Wald and Brown, 1952; Ostroy et al., 1966). This absence in the case of rhodopsin is conceivably due to the lack of a chloride effect in rhodopsin or else the inability to remove chloride from its binding in rhodopsin. I state this as a suggestion because the PMB blue shift is obtained only with the gecko system in the P-Cl^- state, and because chloride added to a PMB-treated pigment leads to a reversal of both the PMB shift and of the chloride deficiency (Fig. 5). If there is any merit to this suggestion, it leads to the idea that the P-Cl^- state has a more open structure, thus unmasking -SH groups that become available to attack by PMB. Despite this, regeneration of the gecko photopigment was not blocked by PMB (Fig. 5), and this is in contrast to the results with rhodopsin and iodopsin whose

[1] The original report (Crescitelli, 1975) in which this blue shift was first described was written before I had discovered the chloride effect so that the relation between the PMB shift and the deficiency of chloride was not mentioned in this report. It is clear, however, that the pigment with its spectral maximum at 507 nm was even then in the P-Cl^- state although I was not aware of it.

regeneration was prevented by this mercurial poison (Wald and Brown, 1952; Wald et al., 1955). This difference in results cannot be a matter of concentration, for rhodopsin inhibition of regeneration was found to begin at a concentration of PMB of 7×10^{-6} M and to be 100% at 8×10^{-5} M (Wald & Brown, 1952). In the experiment of Fig. 5, I employed a concentration of 3×10^{-4} M. To inhibit completely the synthesis of iodopsin, Wald et al. (1955) used a concentration of 1.8×10^{-4} M. Accordingly, at a molarity greater than that which blocks rhodopsin and iodopsin regeneration, the PMB has no effect either on the rate or magnitude of synthesis of the 521-system. All the results with PMB suggest two ideas: (*a*) -SH groups in P-521 are available to attack by PMB, especially in the P-Cl^- state; and (*b*) these groups are not necessary for the primary binding and fit of the retinal to the opsin chromophoric site. For the case of rhodopsin, these -SH groups are either absent or masked and, instead, there are -SH groups in both rhodopsin and iodopsin that do influence the geometry of the chromophoric site. Thus, although the gecko pigment and iodopsin have a number of properties in common (Bliss, 1946; Wald et al., 1955; Matsumoto et al., 1975; Crescitelli, 1977 *a*), the ability to regenerate or not to regenerate in the presence of PMB indicates some not yet identified difference in structure of the opsin chromophoric site in iodopsin and the gecko pigment.

The picture that appears to be emerging for the gecko 521-opsin is that of a molecule with at least four sites that determine and modulate the color of the chromoprotein: (*a*) the primary site of the Schiff base, (*b*) the conformational features that determine the specific stereospecificity, (*c*) the unmasked -SH groups involved in mercaptide formation that are important in the detailed secondary interaction between opsin and the retinal, and (*d*) the ionic site to which chloride (or bromide) reversibly attach to influence the secondary interaction. Site *d* shows no obvious interaction with sites *a* and *b* for the system regenerates as well, and has the same stereospecificity whether or not site *d* is occupied by chloride. Site *d* appears to have some link to site *c*, for the PMB-effect is reversed by chloride and occurs best in the P-Cl^- system.

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