The Photosensitive Retinal Pigment System of *Gekko gekko*

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ABSTRACT Retinal extracts of *Gekko gekko* were found to contain two retinene$_1$ photopigments, one with maximum absorption at about 521 mμ, the second with a maximum in the region of 478 mμ. These pigments were assayed by the method of partial bleaching and their spectral characteristics studied by examining their difference spectra. The 478 mμ pigment was present in the extracts as 8 per cent of the total photopigment concentration. The two pigment systems were shown to be biochemically independent and to have different properties. Unlike the 521 mμ pigment, for example, the 478 mμ pigment was found to resist the action of NH_2OH and, within the cells, to be unaffected by sucrose solutions. These solutions destroyed or altered the 521 system so that extracts of sucrose-treated retinae were found to contain significantly less 521 photopigment. In digitonin solution the 521 pigment was unaffected by sucrose treatment. Both pigments were extracted from separated, washed outer segments and so are considered to be visual pigments. This dual system accounts for the spectral sensitivity of this gecko as determined by Denton. A search was made, but no evidence was secured for the presence of a photopigment absorbing at longer wavelengths. Electoretinographic data suggest, however, that an elevated sensitivity at longer wavelengths occurs in some geckos so that a continued search is justified for a third photopigment.

The chemical basis for scotopic vision in terrestrial vertebrates is normally considered to be a retinene$_1$ photopigment with an absorption maximum at wavelengths around 500 mμ. Denton (1, 2), however, reported that the spectral sensitivity function of *Gekko gekko* was not at this wavelength but some 20 to 30 mμ nearer the red end of the spectrum. Crescitelli (3, 4) suggested that this was due to the nature of the visual pigment which, in geckos, is not classical rhodopsin but another retinene$_1$ pigment located 20 to 30 mμ further toward the red. Crescitelli's conclusion was based, not on a pigment analysis of *Gekko gekko*, but on data secured from a number of other gecko species representing all families of the Gekkonidae. The conclusive analysis with *Gekko gekko* has now been carried out on animals obtained from India and Thailand. In these animals it has been possible to demonstrate the occurrence of a retinal

photopigment with an absorption maximum in the region of Denton's sensitivity curve. This finding, though worthy of publication, would justify no more than a brief note were it not for the fact that digitonin extracts of the retina were found to contain an additional photopigment with an absorption maximum in the blue-green region of the spectrum. Indications of such pigments have been noted in other species of geckos (4, 5) but limitations in number of available animals made it impossible to carry out the crucial controls necessary to establish the existence of these pigments. *Gekko gekko* was procured in sufficient numbers so that most of the experiments were done which are necessary for such a definitive analysis. It was proved that the retina of *Gekko gekko* contains at least two photopigments which are probably visual pigments, one absorbing maximally at about 521 mμ; the second, present in smaller quantities in the extracts, absorbing maximally in the region of 478 mμ. It is the main purpose of this report to give the analytical evidence which supports this statement.

MATERIALS AND METHODS

The lizards, purchased from a dealer, were first dark-adapted for at least an hour and were then cooled to permit easy handling of these biting animals. In the dark room, illuminated only by a dim, deep-red light, the spinal cords were cut at the neck and the eyes were excised after cutting away the spectacles. After washing away the blood from each eye, the cornea, iris, and lens were removed. Pressure on the posterior half of the eye made it possible to extrude the retina and to pick it out with blunt forceps. The retinae were then placed in 4 per cent aluminum potassium sulfate for 2 to 8 hours after which they were washed once with distilled water and once with borate-KCl buffer (pH 8.2). The photopigments were then extracted into 2 per cent digitonin (pH 8.2). Analysis of the extracts was made in the usual way employing a Beckman DU spectrophotometer to determine the optical densities and a Bausch & Lomb monochromator for bleaching the extracts according to Dartnall's method of partial bleaching. The light from the exit slit of the monochromator was first passed through the appropriate interference filter (Baird) before being allowed to strike the extract. All measurements and bleachings were made with the solutions maintained at 20°C ± 1°. Deviations from some of the above procedures were necessary on numerous occasions in order further to examine specific points which came up during the investigation. Twelve extracts were prepared during this study. Each extract, depending on its volume, was subjected to one or more analyses. The extract number (Tables I and II) is indicated by Roman symbols and the specific aliquot of each extract is given by Arabic symbols. Extracts X_a and X_b represent separate extracts run concurrently, X_a being the control for X_b.

ANALYTICAL RESULTS

A. *Histological Considerations*

Examinations of the retina of *Gekko gekko* revealed a typical gecko morphology. The visual cells consist of both single and double rods arranged in layers, the

rows of single cells alternating with the rows of double cells. The outer segments are large (5 to 8 μ by 30 to 40 μ) and cylindrically shaped. These are, in general, the characteristics of the outer limbs of other nocturnal geckos which have been shown to contain considerable quantities of visual pigment (4). There are no oil droplets in the inner segments of either the single or the double rods. The nuclei of the outer nuclear layer exceed in number the nuclei of the ganglion cell layer by a factor of about 3. In many respects this retina conforms to the description given by Tansley (7) for the retina of *Hemidactylus turcicus.*

B. *The Density Spectra*

All extracts were found to contain photosensitive pigment. The density spectrum (Fig. 1) of a relatively pure and thermally stable extract (III) shows the maximum density to be at about 518 mμ, the minimum density at 419 mμ, and a ratio of density at minimum to density at maximum of 0.32. This spectrum is that of a relatively pure photopigment solution and the long wavelength portion (520 to 700 mμ) is probably a correct representation of the spectrum of the photopigment system in this extract. The short wavelength section (below 500 mμ) is probably the spectrum of both the photopigment and of yellow impurities. The latter cause the increase in density at wavelengths below 420 mμ. The contribution by the yellow impurities is small down to about 480 mμ but is increasingly significant at the shorter wavelengths. This extract, like the others, was bleached when exposed to light of appropriate wavelength. The density loss between 440 and 700 mμ which resulted when the extract was exposed to non-isomerizing light (660, 560 and 540 mμ) is given by the dashed line (curve 2) in Fig. 1. This difference spectrum agrees perfectly with the density spectrum (curve 1) from 520 to 700 mμ, indicating that this long wavelength portion of the density spectrum is indeed the spectrum of the photopigment system and is unaffected by any photostable colored impurities. At wavelengths shorter than about 500 mμ the density and difference spectra are significantly different, the disparity increasing as the wavelength decreases. The true absorption spectrum of the photopigment system in extract III would therefore be between the density spectrum, which is too high at shorter wavelengths, and the difference spectrum, which is too low.

Analytical data for the density spectra of all extracts are summarized in Tables I and II. These solutions were of varying degrees of purity, as is indicated by the wide range of values for the ratio of density at minimum to density at maximum. The wavelength for maximum density varied in these several extracts from 499 to 519 mμ. An attempt was made to prepare an especially pure photopigment solution by separating off the outer segments in 40 per cent sucrose solution and then making an extract of these (V-1). Surprisingly—and unlike the retinae of other vertebrates with which I have worked

—the *Gekko* retina did not yield purer extracts by this method. In fact, treatment of the tissue with sucrose solution resulted in relatively impure solutions. This point is illustrated by analysis VI-1 in which 6 retinae were first treated with 40 per cent sucrose solution and then extracted by the usual procedure. The extract so obtained was unusually impure (a ratio for density-minimum to

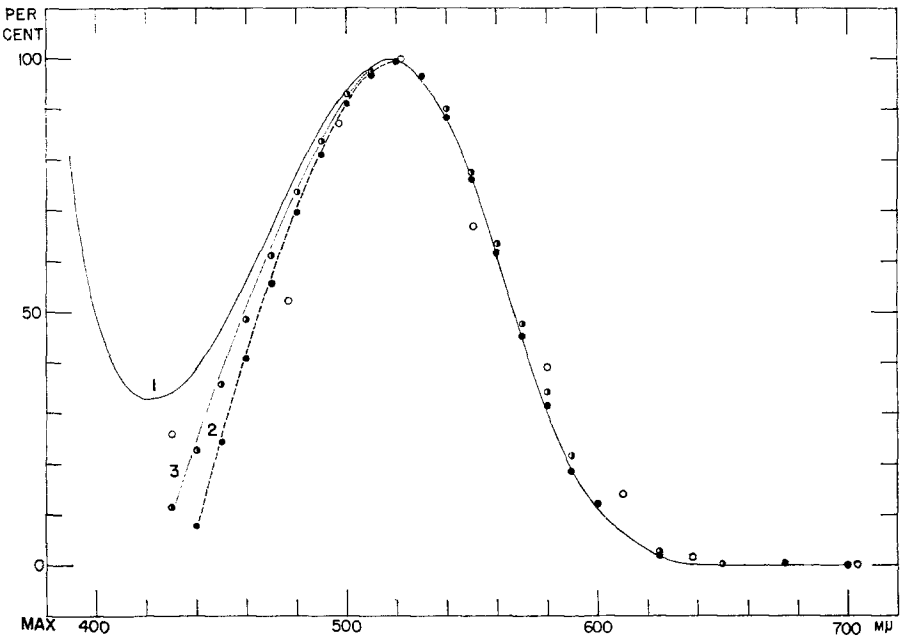

FIGURE 1. Comparison of density spectrum, difference spectra and spectral sensitivity. Curve 1 (full line): density spectrum of extract III, aliquot 2 (analysis III-2). Curve 2: loss of photopigment obtained by exposing extract III to light in three successive stages: 660 mμ for 470 minutes, 560 mμ for 55 minutes and 540 mμ for 45 minutes. Curve 3: loss of pigment in presence of NH_2OH (analysis II-3). Curve 3 gives the total pigment loss resulting from NH_2OH (in the dark) and from an exposure to light in 2 stages; *i.e.*, 640 mμ for 212 minutes and 540 mμ for 57 minutes. Explanation in text. Open circles are Denton's values for spectral sensitivity, expressed in quantum terms. All maxima are scaled to 100 per cent figure to facilitate comparison. Abscissa, in this and all figures (except Fig. 3), in millimicrons.

density-maximum of 0.91) owing to the presence of substances absorbing in the blue and violet regions. The experiments with sucrose also revealed a special effect on the visual pigment system of *Gekko gekko*, which will be described later in this report. In my experience the retinae of many vertebrates, unless first treated with potassium alum, yield impure and often thermally unstable solutions of photopigments. The presence of blood is often a problem in such preparations. In the case of the *Gekko* retina, however, it was possible to obtain

TABLE I

SPECTROPHOTOMETRIC CHARACTERISTICS OF RETINAL EXTRACTS OF *GEKKO gekko*

Analysis-extract and aliquot No.	No. of retinae	Digitonin solution	Density spectra			Difference spectra: Pigment 1		Difference spectra: Pigment 2		Pigment 2	Remarks—special conditions; schedule of bleaching
		ml	λ *max*	λ *min*	$\frac{D_{min}}{D_{max}}$	λ *max*	D_{max}	λ *max*	D_{max}	*per cent**	
I-2	8	1.2	516	428	0.46	522	0.248	478	0.016	6.0	631, 606, 501, white
I-3	8	1.2	No partial bleaching done								NH_2OH caused loss of pigment. 24 hrs. later 520, then white
I-4	8	1.2	514	432	0.49	518	0.178	482	0.022	11.0	660, 540, white
II-1	12	1.2	519	426	0.39	521	0.388	474	0.033	7.8	677, 640, 540
II-2	12	1.2	516	428	0.48	521	0.390‡	475	0.013	3.2§	400, 640, 540
II-3	12	1.2	518	429	0.49	521	0.382‖	478	0.031	7.5	NH_2OH, 640, 540
I + II¶	—	—	517	428	0.49	520	0.330‡	470	0.014	4.0§	400, 640, 540
III-1	12	1.5	516	426	0.41	520	0.284	482	0.027	8.6	709, 691, 660, 540
III-2	12	1.5	518	419	0.32	521	0.271	482	0.022	7.5	660, 560, 540, 400
III-3	12	1.5	515	424	0.36	521	0.266	472	0.030	10.1	NH_2OH, 660, 540, 400
IV-1	6	0.7	507	442	0.78	520	0.395	468	0.028	6.6	No alum; 660, 640, 540
IX-1	10	1.5	512	441	0.73	522	0.265	482	0.028	9.5	Preparation made in blue light; 691, 660, 640, 520

* Percentages computed from density losses of comparable extracts without reference to molar extinction coefficients which are not known.

‡ This figure includes a small amount of pigment 2.

§ This figure includes only amount recovered by final bleach.

‖ 0.275 due to NH_2OH and 0.107 due to 640.

¶ Remnants of extracts I and II combined.

TABLE II

SUCROSE EFFECT*

Analysis-extract and aliquot No.	Density spectra			Difference spectra: Pigment 1		Difference spectra: Pigment 2		Pigment 2	Remarks—special conditions; schedule of bleaching§
	λ *max*	λ *min*	$\frac{D_{min}}{D_{max}}$	λ *max*	D_{max}	λ *max*	D_{max}	*per cent*	
V-1‡	Very impure			521	0.026	480	0.018		40 per cent sucrose, rods isolated; 640, 420
V-2‡	Very impure			523	$\frac{0.035}{0.061}$	478	$\frac{0.006}{0.024}$	28.2	40 per cent sucrose, tissue remnant; 640, 520
VI-1	499	450	0.91	522	0.066	475	0.023	25.8	40 per cent sucrose; 640, 540
VII-1	Impure			525	0.045	479	0.024	34.7	40 per cent sucrose, tissue illuminated; 640; extracted; 640, 540, 440
VIII-1	516	432	0.52	524	0.300	477	0.022	6.8	Control: no sucrose; 640, 540, 520
VIII-2	517	433	0.50	522	0.154	472	0.011	6.6	Pigment extract treated with 100 per cent sucrose solution; 640, 520
X_a-1‖	509	443	0.78	522	0.156	477	0.015	8.7	Control: no sucrose; 640, 540, 520
X_b-1‖	500	445	0.83	520	0.072	477	0.016	18.1	40 per cent sucrose; 640, 520
XI-1	506	435	0.66	521	0.082	475	0.015	15.4	Isotonic sucrose; 640, 520

* Each analysis was of 6 retinae extracted with 0.6 ml digitonin solution.

‡ Rods separated out by sucrose flotation; V-1 is rod extract; V-2 is extract of retinal residue

§ Sucrose solutions made up as grams sucrose per 100 ml of solution.

‖ X_a and X_b are separate extracts but run simultaneously to have a concurrent control.

a stable and relatively pure extract (IV-1) without the use of alum, though this reagent did improve the purity without altering the nature of the results.

C. *Partial Bleaching: The 521 Pigment*

After an extract was exposed to light of relatively long wavelength, spectrophotometric measurements showed only slight losses of photopigment. The difference curves thus obtained gave no evidence for the presence in the extracts of such pigments absorbing at longer wavelengths. Instead, indications were noted of a photopigment characterized by an absorbtion maximum in the region of 520 mμ. A typical result (Fig. 2) illustrates the outcome of exposing an extract (III) to light at 709 mμ for 211 minutes and then to light at 691 mμ for 110 minutes. Only a very small change occurred in the density spectrum as a result of this prolonged exposure, the effect being indicated by the difference spectrum (curve 1) which indicates the loss of a pigment with maximum density at about 520 mμ and the formation of a product with maximum at about 370 mμ. The density loss at maximum was about 0.012 (scale A) while the density gain of the product at maximum was about 0.011. No evidence was secured for the presence of a photopigment absorbing at longer wavelengths. When next the extract was exposed to light at 660 mμ for 120 minutes there resulted a very large loss (0.21) of a pigment with maximum at about 520 mμ and the formation of a product with maximum at about 380 mμ (curve 2, scale B). The similarity of the two difference curves (curves 1 and 2) suggests that one and the same photopigment was involved in the bleachings, first with light at longer wavelengths, second with light at 660 mμ.

Bleaching a visual pigment extract in the presence of added NH_2OH has the advantage that the oxime which is then formed, as the result of the reaction of NH_2OH with retinene, absorbs at wavelengths shorter than is the case for the products of bleaching formed in the absence of NH_2OH. The resulting difference spectrum is therefore less influenced, at shorter wavelengths, by the products of bleaching and is thus analytically more useful. This reagent must be employed with care, however, since it has the tendency to destroy some retinal photopigments. I have noted this action in the case of several gecko pigments. The pigment of *Gekko gekko* is one of these. In order to secure a difference spectrum in the presence of NH_2OH it was necessary to proceed in the following manner: the density spectrum of a 0.5 ml. sample of a retinal extract (II) was first determined. It was similar to that of Fig. 1 (curve 1). A drop of freshly neutralized NH_2OH solution (0.1 M) was then added to the sample. The densities at 4 critical wavelengths (700, 520, 365, 410 mμ) were read immediately after adding the NH_2OH and at intervals for 70.6 hours thereafter. At no time during this procedure was the sample exposed to light

except for the brief period required for the addition of the NH_2OH—and this was done by aid of the deep red light of the dark room— and for the measurements of the optical densities with the spectrophotometer. During the 70.6 hour period the density at 700 mμ remained low and unchanging

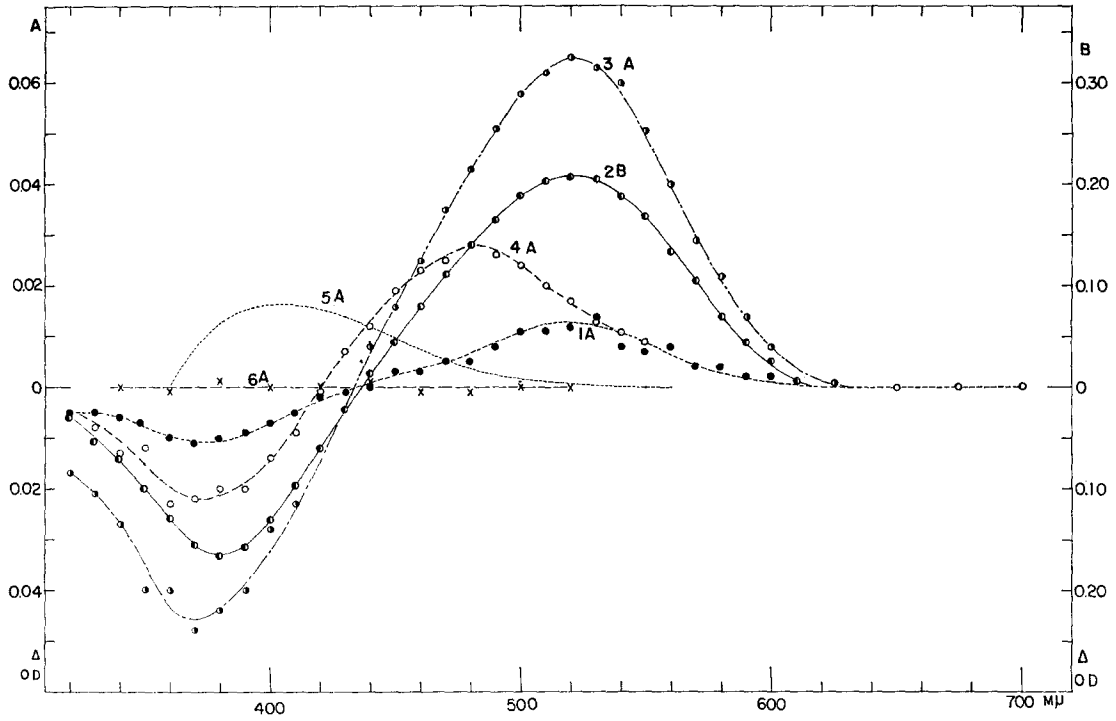

FIGURE 2. Results of analyses III-1, I-2, I-3 utilizing the technique of partial bleaching with non-isomerizing and isomerizing light. Ordinate scale A (0 to 0.06) applies to curves 1, 3, 4, 5, 6. Ordinate scale B (0 to 0.30) applies to curve 2. All curves are difference spectra with values above 0 indicating pigment loss and values below 0 indicating pigment gain. Curve 1: pigment change resulting from exposure to light at 709 mμ for 211 minutes and to 691 mμ for 110 minutes. Curve 2: change after exposure to 660 mμ for 120 minutes. Curve 3: after further exposure to light at 660 mμ for 245 minutes. Curve 4: after exposure to light at 540 mμ for 35 minutes. Curve 5 (analysis I-2): exposure to white light for 10 minutes after all photopigment had been bleached as in curves 1 to 4. Curve 6 (analysis I-3): NH_2OH used to show lack of effect of white light (30 minute exposure) after all photopigment had been bleached.

(curve 4, Fig. 3), indicating the long term thermal stability of this sample. The density at 410 mμ (curve 2), a wavelength which was initially guessed to be near the isosbestic point of the system, remained truly isosbestic up to about 1500 minutes but thereafter increased in density by a small amount. At 520 and 365 mμ (curves 3 and 1) the densities changed progressively, that at 520 mμ decreased, while inversely, and with the same time course—as

was proved by plotting the *changes* in density as a function of time without regard to direction of change— that at 365 mμ increased. In other words, NH_2OH, in the dark, caused the loss of a pigment and the parallel appearance of a product absorbing at shorter wavelengths. In so far as the color change in the region of 520 mμ was concerned, the action of NH_2OH was similar to bleaching by light at wavelengths 600 to 660 mμ. This similarity is revealed by the following: when the destruction of visual pigment had reached a minimal rate at 4240 minutes (Fig. 3) the density spectrum was once again determined in order to ascertain, by means of the difference curve, the nature of the NH_2OH action. This difference spectrum (curve 1, Fig. 4) illustrates the point that NH_2OH had destroyed a pigment with density maximum at 521 mμ and that, in addition, a colored product with maximum at 350 to

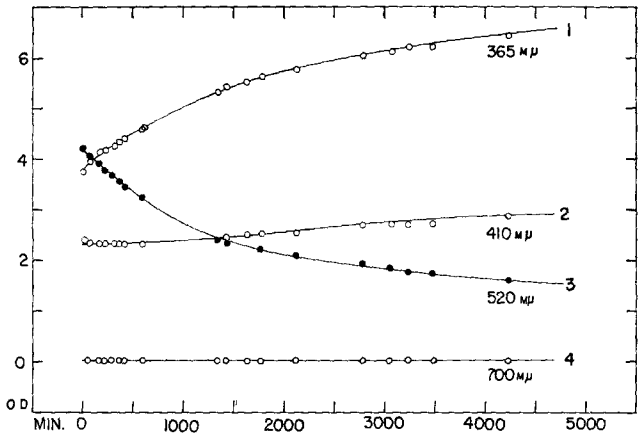

FIGURE 3. Time course of destruction of photopigment (in the dark) by NH_2OH (analysis II-3). Curves 1, 2, 3, 4: changes in optical density (OD) as a function of time (minutes) at wavelengths 365, 410, 520, and 700 mμ respectively.

360 mμ had been formed. To compare this NH_2OH effect with the action of light, the sample, after determination of the density spectrum, was exposed for 212 minutes to light at 640 mμ. This caused bleaching, the result of which was the difference spectrum (curve 2, Fig. 4) which indicates the loss of a pigment with maximum at about 520 mμ and the appearance of a product with maximum at about 370 mμ. This pigment loss was almost entirely due to light, for the density loss at 520 mμ was 0.107 and, from the kinetics of the NH_2OH effect (curve 3, Fig. 3) the photopigment loss expected from the action of NH_2OH in the dark during the period of the light experiment, was not more than 0.03. The total loss of 521 pigment (curves 1 and 2, Fig. 4) which occurred from the action of both NH_2OH and of light was 0.382. This is practically identical with the total photolytic loss in another sample of the same extract (analysis II-1) bleached without the use of NH_2OH, which

yielded a density decrease of pigment 521 of 0.388 (Table I). In other words, both NH_2OH and light produced results which were qualitatively and quantitatively similar. This conclusion justified the addition of the two difference spectra (curves 1 and 2, Fig. 4) and permitted an accounting of the entire photopigment situation in these extracts—as will be discussed later (section D).

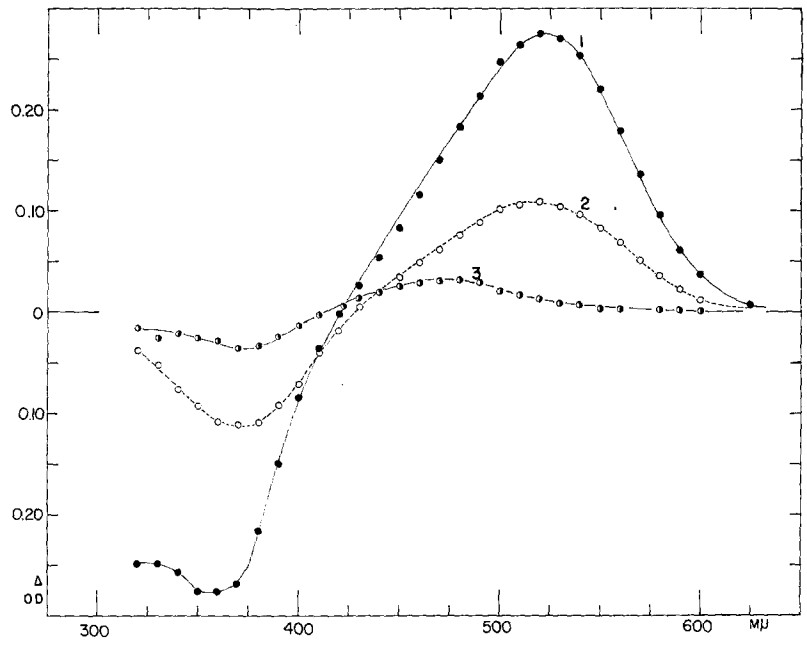

FIGURE 4. Difference spectra resulting from action of NH_2OH in the dark and after partial bleaching (analysis II-3). Curve 1: pigment loss and gain in dark. Curve 2: same after exposure to light at 640 mμ for 212 minutes. Curve 3: same after exposure to light at 540 mμ for 57 minutes. Carried out as explained in text.

The 521 pigment of *Gekko gekko*, like all the gecko pigments already studied (4), appears to be a retinene$_1$ chromoprotein. This conclusion is suggested by the spectral location of the product peak. This location is at 375 to 380 mμ for extracts bleached without NH_2OH and at about 370 mμ in extracts bleached with this reagent. As already indicated (4) these spectral characteristics are those of retinene$_1$ systems. Retinene$_2$ pigments have product peaks at 400 to 405 mμ without NH_2OH and at about 388 mμ with this amine. This conclusion was convincingly supported by the agreement between the difference spectrum of the product peak in analysis I-3 (Table I), bleached in the presence of NH_2OH, and the spectrum of the oxime formed when NH_2OH was added to a solution of all-trans retinene$_1$ in 2 per cent digitonin

(pH 8.3). This comparison is not included in the graphic results of this report because it is similar to the result already included in Fig. 3 of a previous paper by Crescitelli (3).

D. *Partial Bleaching: The 478 Pigment*

After all the 521 pigment was removed by exhaustive bleaching with light at 660 or 640 mμ, further exposure to light at 540 or 520 mμ resulted in a small but unique change in density which was never the same as that resulting from exposures to light at longer wavelengths. This unique action involved a selective loss of density maximal in the region of 478 mμ and a product peak with maximum at 370 to 380 mμ. The nature of this effect is demonstrated in a typical analysis (Fig. 2) in which 3 initial successive exposures to light at longer wavelengths resulted in 3 difference curves (curves 1, 2, 3) all of which involved the bleaching of 521 pigment. The sample was then illuminated for 35 minutes with light at 540 mμ and this resulted in a difference spectrum (curve 4) which was clearly unlike the initial 3 results. The maximal density loss was at about 480 mμ but the product peak was in the same region as for the 521 pigment. A second exposure to light at 540 mμ for 50 minutes produced no further change in density indicating that all of the 478 pigment had been bleached. A total of 12 analyses similar to that of Fig. 2 were performed, first removing the 521 pigment as described and then bleaching out the second pigment. The 478 pigment was found to be present in every extract. In those analyses which did not involve the use of sucrose solution, the proportion of the 478 pigment was 7 to 10 per cent of the total photopigment concentration, the mean figure being 8 per cent.

The finding of a 478 difference spectrum needs to be critically examined in the light of several possible interpretations. One of these is whether or not this result was the outcome of an isomerizing action by light on the products of bleaching of the 521 pigment. A detailed examination was made of this point and this interpretation was rejected for the following reasons: isomerizing effects were distinctly different from the results of bleaching with light at 540 or 520 mμ (Fig. 2). Both the form and location of the difference curve (curve 5) obtained after isomerizing a bleached extract were unlike the 478 effect (curve 4). Moreover, the 478 difference spectrum was quantitatively and qualitatively constant from analysis to analysis whereas isomerizing actions varied from extract to extract, presumably related to the varying degrees of extract purity. In addition, NH_2OH either abolished or altered the isomerizing action (curve 6, Fig. 2) but it did not influence the form or quantitative recovery of the 478 pigment (curve 3, Fig. 4; analysis II-1 and II-3, Table I).

There is also the interpretation that pigment 478 was the result of bleaching the 521 pigment. This hypothesis may be eliminated by demonstrating the

quantitative independence of the two pigments under conditions where the concentration of either one was initially reduced. The following data are adduced to support this conclusion:

(*a*) In one of the analyses with NH_2OH (Fig. 4) the total recovery of 521 pigment was 0.382. Of this figure 0.275 was due to destruction in the dark by NH_2OH and 0.107 was the result of photobleaching. The amount of 478 pigment was 0.031. This is the same figure as for analysis II-1 (Table I) in which all the 521 pigment was destroyed by light. In other words, the quantity of 478 pigment found in the extract was unrelated to the quantity of 521 pigment which was photolyzed. Incidentally, the quantitative agreement in recoveries of 478 pigment in analyses with and without NH_2OH suggests the occurrence of a preferential destruction by NH_2OH on the 521 system.

(*b*) Initial exposure of an extract to light at 400 mμ led to lower final recoveries of the 478 pigment suggesting that this pigment was initially present in the extract and was not related to the amount of 521 pigment which was bleached. Thus in two such analyses (II-2, I + II) the data (Table I) showed the amount of 478 pigment at terminal recovery to be 3.2 and 4.0 per cent of the total photopigment concentration. These figures, secured with total pigment concentrations at normal levels, were significantly smaller than in those analyses which omitted the initial exposure to light at 400 mμ (Table I).

(*c*) A third type of evidence was discovered in trials with the sucrose flotation technique, which separates the outer segments of the visual cells from the other retinal tissue. This technique was employed in attempts to get purer solutions of visual pigments and in order to determine whether or not the 478 pigment was present in the outer segments, the correct location for visual pigments. These attempts led, not only to less pure solutions, but also revealed a preferential destruction of pigment 521 so that final recoveries of pigment 478, rather than being 6 to 10 per cent as usual, were 18 to 28 per cent of the total photopigment concentration. Thus in one experiment utilizing the retinae of 3 geckos, extraction of the separated outer segments (V-1) and of the retinal residue (V-2) which remained after removal of the outer limbs led to results (Table II) which showed the densities of pigment 521 to be 0.026 for the outer limbs and 0.035 for the retinal residue, a total of 0.061. This is considerably less than the results of a control analysis (VIII-1) in which the retinae of 3 geckos were extracted with the same volume (0.6 ml) of digitonin solution, and the procedure was the same except for the omission of the sucrose treatment. In this case the density loss for the 521 pigment was 0.300. In contrast, the 478 pigment loss was the same for the control analysis (0.022) and for the sucrose-treated extract (0.024; outer segments 0.018 + retinal residue 0.006). The sucrose treatment had destroyed or rendered unavailable for extraction some 67 per cent of the 521 pigment while leaving unaffected all the 478 pigment. An important outcome of this

analysis was the finding that about 75 per cent of the 478 pigment appeared in the extract of the outer segments, a result in support of the hypothesis that pigment 478 is indeed a visual pigment.

The results of another analysis in this sucrose treatment series are graphically pictured in Figs. 5 and 6. The density spectrum (curve 2, Fig. 5) of an extract of sucrose-treated retinae, in comparison with the result (curve 1, Fig. 5) of a control analysis (VIII-1), showed that sucrose led to a less pure solution and one which contained much less photopigment. This deficit was accounted for entirely by a reduction in the 521 system (Fig. 6) since the

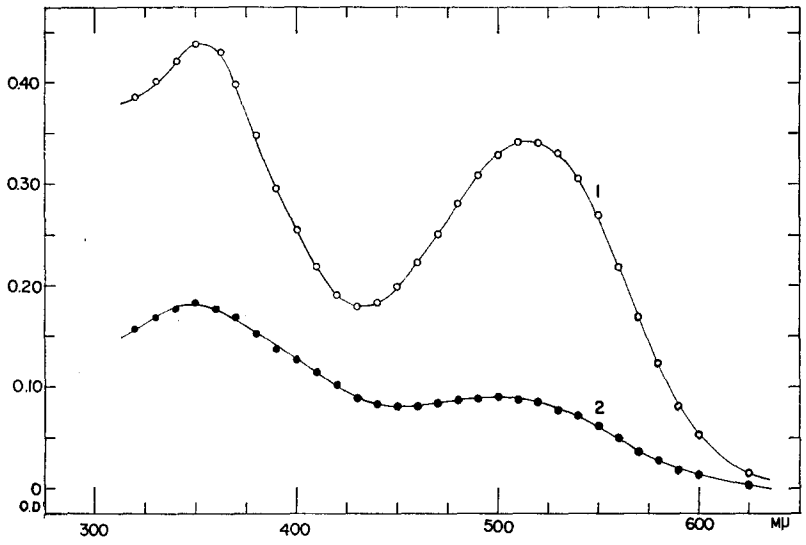

FIGURE 5. Density spectra to show effect of treating retinae with sucrose solution before extracting the visual pigments. Curve 1: untreated control analysis (VIII-1). Curve 2: result of treating with 40 per cent sucrose solution before extraction (analysis VI-1). All other procedures as for the control.

521 pigment losses were 0.300 for the control and 0.066 for the experimental extract whereas the 478 pigment losses were identical at respective values of 0.022 and 0.023. Thus while the 521 pigment showed a 78 per cent deficit the 478 system was unaffected.

At this point the sucrose effect is relevant because it permitted the decisive demonstration that the quantity of 478 pigment recoverable by a terminal bleaching with light at 540 or 520 mμ was independent of the amount of 521 pigment in the extract. This hypothesis of the biochemical independence of these two pigment systems was put to an extreme test in an analysis (VII-1) in which the retinae of 3 geckos were suspended in 40 per cent sucrose solution and then, while thus suspended, were exposed to light at 640 mμ for 4

hours in order to reduce as much as possible the concentration of the 521 pigment. Following this light exposure an extract was prepared in the usual manner employing 0.6 ml digitonin solution. The density loss for the 521 pigment was found to be 0.045 (Table II) while for the 478 pigment the loss was 0.024. Compared to a control analysis (VIII-1) this procedure demonstrated that the concentration of the 521 system may be reduced by 85 per cent without affecting the quantitative recovery of the 478 pigment. These two systems are therefore considered to be independent biochemical entities both of which are present in the outer segments of the visual cells. The spectral locations of the product peaks with NH_2OH (Fig. 4) and without NH_2OH (Fig. 2) suggest that these two are retinene$_1$ photopigments.

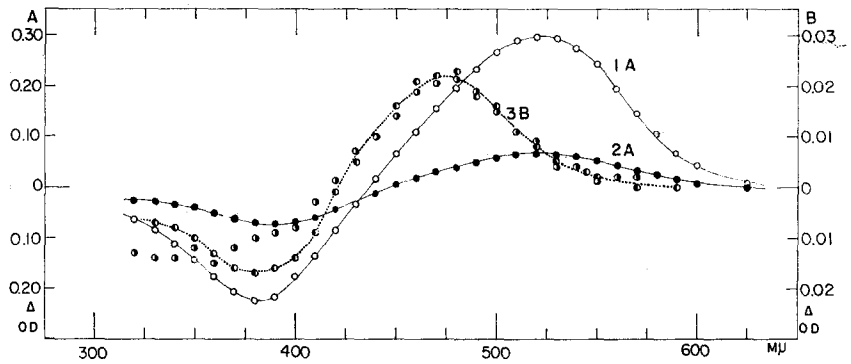

FIGURE 6. Effect of sucrose treatment (analyses VIII-1 and VI-1). Ordinate scale A (0 to 0.30) applies to curves 1 and 2; scale B (0 to 0.03) applies to curve 3. Curve 1: pigment loss and gain in untreated control extract after exposure to light at 640 mμ for 241 minutes. Curve 2: result of exposing the extract of sucrose-treated retinae to light at 640 mμ for 316 minutes. Curve 3: pigment loss and gain for both control and experimental analyses. The control (shown as ◑) was exposed, after total removal of the 521-pigment, to light at 540 mμ for 60 minutes. The experimental (shown as ◐) was similarly exposed to light at 540 mμ for 60 minutes. Further explanation in text.

E. *Is There a Photopigment at Longer Wavelengths?*

All previous analyses were carried out at room temperature and deep red light was employed as illumination in the procedures of removing the retinae, processing and extracting them, and in loading the cuvettes with the extracts. It is possible that the conditions which were employed were not propitious for the preservation of a photopigment absorbing at longer wavelengths. Consequently, one experiment was made utilizing for illumination light which had first passed through an interference filter with peak transmission at 424 mμ. The retinae of 5 geckos were dissected out, washed twice in cold (5°C) water and then extracted with 1.5 ml of cold 2 per cent digitonin solution at pH 7.0. Thus all conditions, *i.e.* red light, higher temperatures, alum,

alkaline solutions, were avoided in an effort to preserve a pigment absorbing in the red region. The extract was kept for 3 days in the freezing compartment of the refrigerator and was then loaded into a cuvette, utilizing the blue light for visibility, following which it was analyzed by the method of partial bleaching. Exposure of this extract to light at 691 mμ for 55 minutes resulted in no loss of optical density. Illumination with light at 660 and 640 mμ (in two stages) each resulted in the typical 521 difference spectrum with no evidence of excess density loss at longer wavelengths. This extract also yielded a typical 478 curve, the 478 pigment constituting 9.5 per cent of the total photopigment. There was no evidence from this analysis (IX-1) that either the 521 or the 478 pigments were deficient in amount in this extract prepared with blue light as illumination.

F. *The Sucrose Effect*

The sucrose effect was an incidental observation and was made use of in justifying the biochemical independence of the two pigment systems of this retina. The mechanism of action of sucrose in these experiments is unknown and remains a subject for future inquiry. Nevertheless, some observations were made which are of possible interest in connection with such an inquiry. A hypertonic sucrose solution was not the essential condition for this effect as analysis XI-1 indicates. For this test the retinae of 3 geckos were placed in isotonic (0.22 M) sucrose solution for 17 hours before processing and extracting with 0.6 ml digitonin. The extract had both the 521 and the 478 systems, the former with a density loss of 0.082; the latter with one of 0.015. The 478 pigment was 15.4 per cent of the total. This was a real deficit although not as great a one as when 40 per cent sucrose was employed. Another important point about this sucrose effect was that no such action was observed when the 521 pigment was in solution in digitonin. This point is illustrated by analysis VIII-2 in which a 0.2 ml sample of the extract was mixed with 0.2 ml of a solution of 100 per cent sucrose. The density spectrum, measured 2.5 hours later, was identical—except for the effect of dilution—to another sample of the same extract (VIII-1) which was undiluted. A partial bleaching analysis of the sucrose-treated sample revealed the 521 pigment to have a density loss of 0.154 and that of the 478 pigment to have one of 0.011. These values are 50 per cent of the corresponding figures for the undiluted control (VIII-1). I have not observed a sucrose effect on any other visual pigment with which I have previously worked but the conditions have not been as favorable as in the *Gekko* retina, in which the presence of a second photopigment, apparently unaffected by the sucrose solution, made the ratio of the two pigments a sensitive index of unusual behavior. It is possible, of course, that some minute impurity in the sucrose, rather than the sugar itself, was responsible for the effect, but this point remains to be investigated.

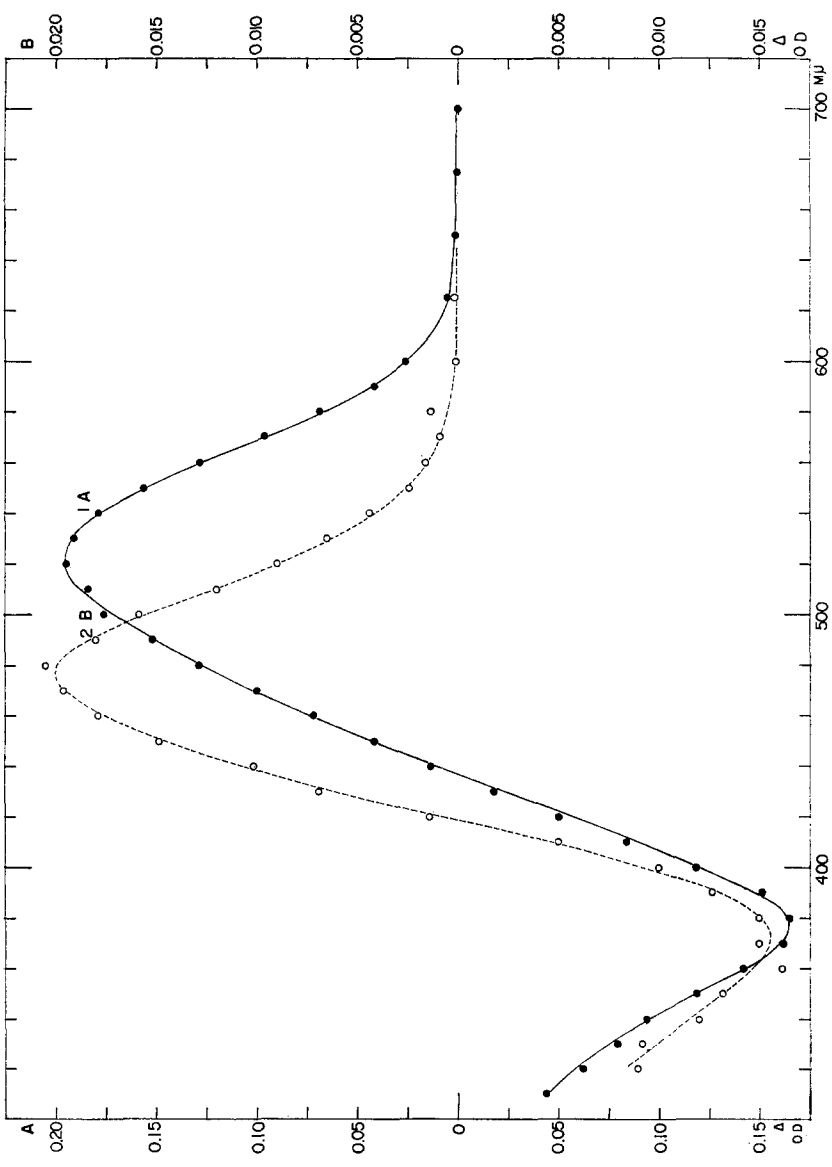

FIGURE 7. Summary of 12 partial bleaching analyses showing mean data for pigment-521 (curve 1) and pigment-478 (curve 2). Ordinate scale A (0 to 0.20) applies to curve 1; scale B (0 to 0.02) applies to curve 2.

DISCUSSION

A. *The Dual Pigment System*

The results of 12 analyses by the partial bleaching technique are summarized in the mean difference curves (Fig. 7) for the two pigment systems found in the extracts. The 521 system (curve 1) is characterized by a spectral

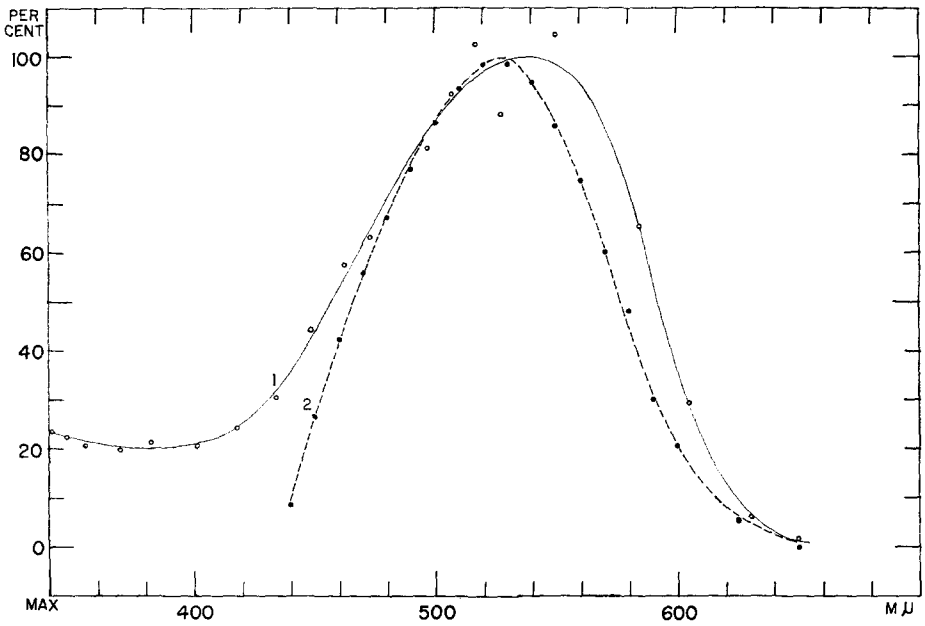

FIGURE 8. *Hemidactylus turcicus.* The spectral sensitivity curve compared with the difference spectrum (total photopigment loss). Sensitivity data (curve 1), expressed in quantum terms, are derived from measurements of the b-wave and were kindly supplied by Dr. E. Dodt. The difference spectrum (curve 2) was constructed from data obtained in this laboratory. The total photopigment content was removed by non-isomerizing light. A similar disparity at longer wavelengths was observed in a comparison of data with *Tarentola mauritanica.*

location for maximal density loss at 521 ± 1 mμ; a density loss of 0.196; and a spectral location for maximum density gain at 377 ± 1 mμ. The corresponding figures for the 478 system (curve 2) are 478 ± 1 mμ, 0.020 and 373 ± 1 mμ. Both these pigments are apparently retinene$_1$ compounds, as are all other gecko retinal photopigments which have so far been examined (4). This is suggested by the spectral locations of the product peaks (Fig. 7) and the other spectral characteristics already mentioned. The dual system of *Gekko gekko* is probably not unusual for geckos. Although a more thorough and more careful analysis was possible with the photopigments of this lizard, indications and evidence of similar duality were noted in *Oedura monilis*, *Hemidactylus*

turcicus, *Hemidactylus frenatus* and *Tarentola mauritanica* (Crescitelli, in press: Mazia and Tyler, General Physiology of Cell Specialization, New York, McGraw-Hill Book Co., Inc.). The present study has more conclusively ruled out isomerization and biochemical artifacts as an explanation for the second pigment.

The results of this paper conclusively confirm for the same species the earlier suggestion (3) that the nature of the gecko visual pigment system was the explanation for Denton's unusual spectral sensitivity curve (2) for *Gekko gekko*. The agreement between Denton's figures and the biochemical data is summarized in Fig. 1. At wavelengths from 500 to 700 mμ the sensitivity data (open circles) are in reasonable accord with the density spectrum (curve 1) and with the two difference spectra (curves 2 and 3). At wavelengths below 500 mμ the true absorption spectrum is undoubtedly in between the density spectrum (curve 1), which is too high at these shorter wavelengths because of the presence of yellow impurities, and the alkaline difference spectrum (curve 2) which is here too low because of the contribution of the colored products of bleaching. Denton's two points at 400 mμ and at 430 mμ are in between these two curves, which is the proper location. Denton's point at 477 mμ is lower than even the alkaline difference spectrum at this wavelength and is probably in error. The two photopigments found in the extracts are very likely visual pigments and their extraction from the isolated outer segments is in accord with this view. Of special interest are the numerous double visual cells which are so characteristic of the retina of this and of other geckos. Does each member of the pair have the same or a different visual pigment? The *Gekko* retina with its large outer segments would appear to be a suitable preparation for the *in vivo* examination of this question. This investigation provides two photopigments for future anatomical allocation.

B. *Possibility of a Third Photopigment*

The failure of the analysis to discover a photopigment absorbing in the long wavelength region of the spectrum is not now considered proof of the absence of such a substance. It is known, for example, that pigments at longer wavelengths tend to be unstable. Chicken iodopsin was found by Wald, Brown, and Smith (8) to be unstable in alkaline solutions and Bridges (9) noted a similar instability for pigment 544 of the pigeon retina. Little success has yet been achieved in attempts to extract visual pigments located at the red end of the spectrum, although electrophysiological data (Granit, 10) tends to suggest the occurrence of such pigments for several vertebrates. In this connection I have compared some biochemical data obtained by me on two other geckos (*Tarentola mauritanica*, *Hemidactylus turcicus*) with the spectral sensitivity functions of these two lizards. The data for the sensitivity were kindly furnished by Dr. E. Dodt and were taken from a paper by Dodt and

Walther (11) on the electroretinogram. For both species the comparison showed an elevated sensitivity at longer wavelengths which was apparently not accounted for by the biochemical curves. This lack of accord at longer wavelengths is illustrated, for *Hemidactylus*, in Fig. 8 which gives the sensitivity function (curve 1) and the difference spectrum (curve 2). In the very region (530 to 700 mμ) where the difference spectrum is an accurate indicator of the absorption spectrum, the sensitivity function departs significantly from the biochemical data. It is noteworthy that no such disparity is seen in the comparison with Denton's sensitivity function (Fig. 1) for the case of *Gekko gekko*. Denton's measurements, based on the response of the pupil, were made with a differential method in which the responses to the test wavelengths were compared with the responses of the pupil exposed to an orange-red light. Such a procedure could have depressed the sensitivity of a "red" receptor and have resulted in a failure to find heightened sensitivity at the longer wavelengths. I am at present engaged in the determination by means of the electroretinogram, of the spectral sensitivity of *Gekko gekko*. All this means that the search for a photopigment at longer wavelengths is still justified in geckos and the possibility still exists for the occurrence of at least a triple retinal photopigment system in some of these nocturnal lizards.

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