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DETECTION OF LIGHT-INDUCED CHANGES OF INTRACELLULAR IONIZED CALCIUM CONCENTRATION IN *LIMULUS* VENTRAL PHOTORECEPTORS USING ARSENAZO III

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SUMMARY

1. The metallochromic indicator dye, arsenazo III, was injected intracellularly into *Limulus* ventral photoreceptor cells to concentrations greater than 1 mM.

2. The absorption spectrum (450–750 nm) of the dye in single dark-adapted cells was measured by a scanning microspectrophotometer. When a cell was light-adapted, the absorption of the dye changed; the difference spectrum had two maxima at about 610 and 660 nm, a broad minimum at about 540 nm and an isosbestic point at about 585 nm.

3. When intracellular calcium concentration was raised in dark-adapted cells previously injected with arsenazo III, the difference spectrum had two maxima at about 610 and 660 nm, a broad minimum at about 530 nm and an isosbestic point at about 585 nm. The injection of Mg^{2+} into dark-adapted cells previously injected with the dye induced a difference spectrum that had a single maximum at about 620 nm. Also, decreasing the intracellular pH of cells previously injected with the dye induced a difference spectrum that had a minimum at about 620 nm. The evidence suggests that there is a rise of intracellular ionized calcium when a *Limulus* ventral photoreceptor is light-adapted.

4. The intracellular calcium concentration, $[Ca^{2+}]_i$, in light-adapted photoreceptors was estimated to reach at least 10^{-4} M by comparing the light-induced difference spectra measured in ventral photoreceptors with

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a standard curve determined in microcuvettes containing 2 mM arsenazo III in 400 mM-KCl, 1 mM-$MgCl_2$ and 25 mM MOPS at pH 7·0.

5. In cells injected to less than 3 mM arsenazo III, light induced a transient decrease in optical transmission at 660 nm (T_{660}). This decrease in T_{660} indicates that illumination of a ventral photoreceptor normally causes a transient increase of $[Ca^{2+}]_i$.

6. Arsenazo III was found to be sensitive, selective and rapid enough to measure light-induced changes of intracellular ionized calcium in *Limulus* ventral photoreceptor cells.

INTRODUCTION

It has been proposed that changes of intracellular calcium concentration, $[Ca^{2+}]_i$, participate in the physiology of light-adaptation of the ventral photoreceptor cells of *Limulus polyphemus* (Lisman & Brown, 1972*a*, *b*). Several lines of evidence support this hypothesis. (1) Increasing $[Ca^{2+}]_i$ directly by intracellular iontophoretic injection mimics two effects of prior illumination of a ventral photoreceptor: the sensitivity to light is reduced and the time scale of the response is shortened (Brown & Lisman, 1975). (2) The reduction of sensitivity after intracellular injection of calcium is local within the cell, similar to the local reduction of sensitivity that can be produced by spatially restricted illumination ('local-adaptation'; Fein & Lisman, 1975). (3) Intracellular injection of the calcium-sequestering agent EGTA (ethyleneglycol bis (β aminoethyl ether) *N*,*N*,*N*′,*N*′-tetra-acetic acid) tends to stabilize $[Ca^{2+}]_i$ and also to prevent light-induced reduction of sensitivity (Lisman & Brown, 1975). (4) Also, increases of $[Ca^{2+}]_i$ normally elicited by light can be detected in ventral photoreceptors using the photoprotein aequorin (Brown & Blinks, 1974). Specifically, light elicits a transient increase of luminescence from aequorin injected into a ventral photoreceptor. The aequorin luminescence reaches a maximum after the peak of the electrical response of the cell and decays to a much lower level during a prolonged stimulus. In sum, these findings suggest that light normally induces a rise of $[Ca^{2+}]_i$ which is a step in the sequence of events underlying light-adaptation.

Quantitative measurements of the time course and magnitude of changes of intracellular ionized calcium concentration ought to elucidate further the mechanism of light-adaptation. However, the studies using aequorin are difficult to interpret quantitatively. The stoichiometry of the Ca–aequorin binding reaction is uncertain and depends on the conditions of measurement (Ashley, 1970; Baker, Hodgkin & Ridgway, 1971; Blinks, 1973; see also the review, Blinks, Prendergast & Allen, 1976). The luminescent reaction sequence is irreversible; therefore, a decline of luminescence might indicate either a decline of calcium concentration or the depletion

of aequorin from some putative intracellular compartment(s). Aequorin is a relatively large molecule (mol.wt at least 20,000 daltons) and may not be freely diffusible into the appropriate intracellular spaces. In addition, the kinetics of the luminescent reaction are slow; the half-time of the rise of luminescence is about 6 msec at 20° C (which is relatively slow by comparison with many physiological events, although not for light responses of ventral photoreceptors). The half-time of decay of luminescence is about 600 msec at 20° C in an excess of calcium. For all these reasons, it is appropriate to re-examine the light-induced changes of $[Ca^{2+}]_i$ in ventral photoreceptors by means of a technique that has a different chemical basis.

In this paper we describe light-induced changes of $[Ca^{2+}]_i$ in ventral photoreceptors detected by the metallochromic indicator dye arsenazo III (2,7-bis[(*o*-arsonophenyl)azo]-1,8-dihydroxynaphthalene-3,6 disulphonic acid). The change in absorption spectrum when arsenazo III binds calcium is at least ten-times faster than the luminescence produced when aequorin binds calcium (Brown, Cohen, DeWeer, Pinto, Ross & Salzberg, 1975). The rapid change in absorption is sensitive enough to detect changes in ionized calcium elicited by depolarizing pulses in a voltage-clamped squid giant axon (Brown *et al.* 1975). In *Limulus* ventral photoreceptors the characteristics of the changes in absorption spectra establish that the dye is both selective and sensitive enough to detect light-induced changes of the intracellular ionized calcium concentration.

METHODS

Ventral rudimentary eyes of *Limulus polyphemus* (Demoll, 1914; Clark, Millecchia & Mauro, 1969) were dissected free of their ensheathing blood vessels and pinned in a Sylgard 184 (Dow Corning, Midland, Mi.) chamber. The eyes were bathed in artificial sea water (designated 10 mM-Ca^{2+} sea water) containing (in mM): NaCl, 425; KCl, 10; $MgCl_2$, 22·9; $MgSO_4$, 25·5; $CaCl_2$, 10; and Tris Cl, 10; at pH 7·8. In those experiments in which calcium ion concentration was changed in the bath, $MgSO_4$ was replaced by equimolar $MgCl_2$ and the $CaCl_2$ was either omitted from the sea water (designated 0 mM-Ca^{2+} sea water) or 40 mM-$MgCl_2$ was replaced by 40 mM-$CaCl_2$ (designated 50 mM-$Ca_2{}^+$ sea water). Replacement of sulphate by chloride itself produces no noticeable changes of the light response of ventral photoreceptors (Millecchia & Mauro, 1969).

The electrophysiological apparatus for recording membrane voltage, voltage clamping or iontophoretic injection of ions has been described (Lisman & Brown, 1971). Micropipette electrodes of the type used for recording membrane voltage, iontophoretic injection of ions, or pressure injection of dyes had resistances of about 5 MΩ when filled with 3 M KCl. For iontophoretic injections, micropipettes were filled with either 1·0 M EGTA, 1·0 M-$MgCl_2$, or a calcium buffer solution (0·09 M-CaOH, 0·1 M Tris base and 0·1 M EGTA; Reuben, Brandt & Grundfest, 1974; Lisman & Brown, 1972*b*). Micropipettes used for pressure injection of dyes were filled with 30 mM solutions of either arsenazo III or phenol red, adjusted to about pH 8 with

KOH. All solutions used for filling micropipettes were filtered immediately before use through a Metricel TM (Gelman, Ann Arbor, Mi.) filter, pore size 0·2 μm.

The volume of solution injected was quantified (Lisman & Brown, 1975; Coles & Brown, 1976) by including $H_2{}^{35}SO_4$ in the solution. After injection, the tissue was partially digested in Pronase (Calbiochem, La Jolla, Ca.) and counted in Aquasol (NEN, Boston, Ma.) in a liquid scintillation counter. The volume of solution injected was calculated from the proportion of c.p.m. in the sample to that in a known volume of the injected solution counted under identical conditions.

Arsenazo III. Arsenazo III (mol. wt. = 776) was obtained commercially (Aldrich Chemical Co., Milwaukee, Wi.). Dye solution, at a concentration of 25 mg/ml., was titrated to about pH 8 and freed of calcium by passage through a column of Chelex 100 (Biorad Labs, Richmond, Ca.) in the potassium form. The dye was precipitated in acid (Budesinsky, 1969*b*, p. 52), filtered under suction and dried in a vacuum desiccator over KOH pellets. The calcium remaining in the dye was determined by atomic absorption spectroscopy to be less than $1{\cdot}2 \times 10^{-5}$ mole Ca per gram of dye.

When arsenazo III binds calcium it changes colour and its peak absorption shifts to longer wave-lengths (Savvin, 1961; Michaylova & Ilkova, 1971; Michaylova & Kouleva, 1973). Difference spectra of the arsenazo III–Ca^{2+} complex are shown in Fig. 1 *A*. The exact shapes of the curves depend on ionic strength, dye concentration, magnesium ion concentration and pH as well as Ca^{2+} concentration. The Ca^{2+} difference spectra exhibit a broad minimum at about 530 nm, two maxima at about 610 and 660 nm and a wave-length, 585 nm, at which there is no change in absorption when the Ca^{2+} concentration changes (the isosbestic wave-length). The selectivity of Ca^{2+}-binding over Mg^{2+}-binding in 400 mM-KCl at pH 7·0 was greater than thirty-fold, at 660 nm. A difference spectrum for Mg^{2+}-binding by the dye is shown in Fig. 1*B*; the curve has a single maximum at about 620 nm. The dye can also indicate changes in pH. As shown in Fig. 1*C* the difference spectrum for a decrease in pH has a minimum at about 620 nm.

Purity of arsenazo III. Commercially available samples of the dye contain substances other than arsenazo III. Because no preparative procedure for purifying the dye was known to us, we ascertained the purity of the acid-precipitated dye by paper chromatography. Dye was applied to the origin of the paper (Whatman 3MM, previously washed with EDTA) at 50 μg/mm and eluted with solvent (*n*-propanol, ammonium hydroxide, water; 45:10:45 v/v) at 20° for 18 hr. The resulting chromatogram contained three coloured bands, brown (relative band speed to front, $R_f \approx 0{\cdot}05$), violet ($R_f \approx 0{\cdot}30$), and pink ($R_f \approx 0{\cdot}36$). Each band was eluted with water and analysed by spectrophotometry.

The contents of each band were mixed with pH buffer (K^+–HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), pH 7·5), to a final buffer concentration of 25 mM. In the absence of divalent cations (2 mM-K^+–EDTA, pH 7·5)

Fig. 1. Difference spectra of arsenazo III measured in 1 cm cuvettes in a Cary 14 (Palo Alto, Ca.) spectrophotometer. *A*. difference spectra of calcium binding with arsenazo III (0·1 mM) made in a solution containing 400 mM-KCl, 25 mM MOPS at pH 7·0 and 1 mM-$MgCl_2$. The concentrations of added calcium (micromolar) are listed beside each spectrum on the Figure. *B*, difference spectrum of magnesium binding with arsenazo III (0·1 mM) made in a solution containing 400 mM-KCl and 25 mM MOPS at pH 7·0 (4 mM-Mg^{2+} spectrum minus 1 mM-Mg^{2+} spectrum). *C*, difference spectrum of arsenazo III (0·1 mM) for a reduction in pH (pH 6·0 spectrum minus pH 7·0 spectrum) made in a solution containing 400 mM-KCl, 1 mM-KCl, 1 mM-$MgCl_2$ and 25 mM MOPS.

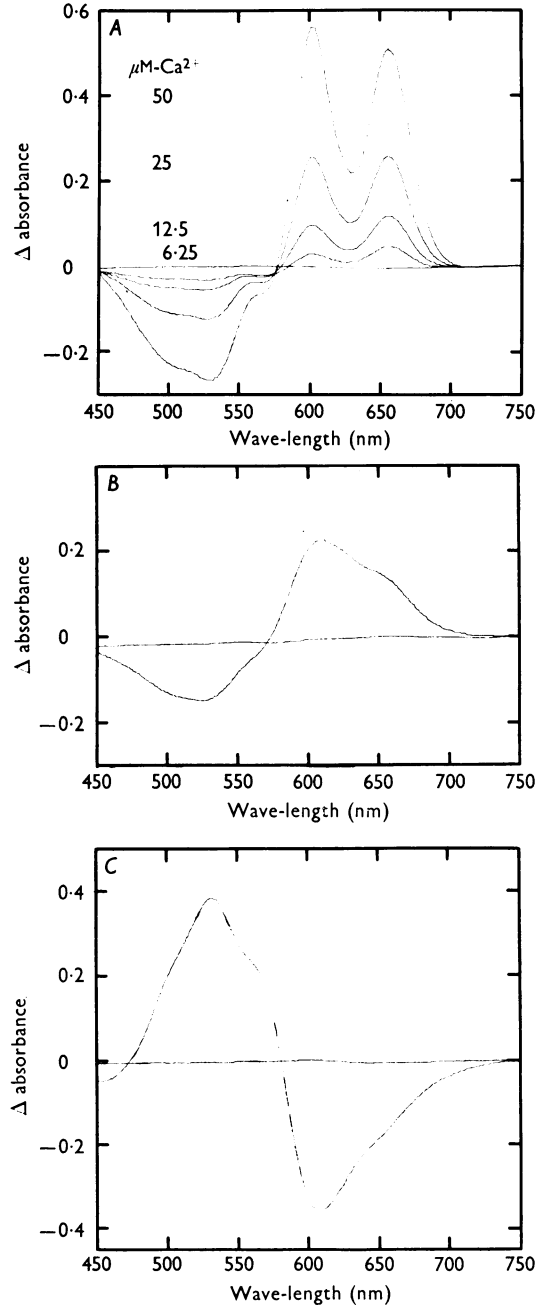

Fig. 1. For legend see facing page.

the material in each band had a single absorption maximum (λ_{max}). To measure approximately equal maximum absorbances for all the bands, it was necessary to dilute the $R_f \approx 0{\cdot}30$ band fifty to a hundred times more than the others. For each band we measured the absorbance at 585 nm (A_{585}) and normalized this quantity to the absorbance at 585 nm of the band of $R_f \approx 0{\cdot}30$, corrected for dilution. The values obtained were: for the band of $R_f \approx 0{\cdot}05$, $\lambda_{max} = 450$ nm, A_{585}/A_{585} ($R_f \approx 0{\cdot}30$) = 5×10^{-3}; for the band of $R_f \approx 0{\cdot}30$, $\lambda_{max} = 550$ nm, A_{585}/A_{585} ($R_f \approx 0{\cdot}30$) = 1·0; for the band of $R_f \approx 0{\cdot}36$, $\lambda_{max} = 515$ nm, A_{585}/A_{585} ($R \approx 0{\cdot}30$) = 3×10^{-3}. For the bands of $R_f \approx 0{\cdot}05$ and 0·36, spectra obtained before and after addition of excess Ca^{2+} were identical; however, the spectrum for the band of $R_f \approx 0{\cdot}30$ changed and gave a difference spectrum with two maxima at about 600 and 650 nm, one broad minimum at about 520 nm and an isosbestic wave-length at about 570 nm. The absorption spectrum and difference spectrum of the contents of the band of $R_f \approx 0{\cdot}30$ are consistent with those of chromatographically pure arsenazo III (Budesinsky, 1969*a*).

The purity of the arsenazo III band ($R_f \approx 0{\cdot}30$) was confirmed using two additional techniques. The arsenazo III migrated as a single band during paper electrophoresis using a 30% (v/v) aqueous solution of glacial acetic acid as the buffer (Savvin, Akimova, Krysih & Davydova, 1970). The arsenazo III also migrated as a single band during anion exchange chromatography with DEAE cellulose adsorbent and a solvent consisting of *n*-butanol, pyridine, glacial acetic acid and water, 3:1:1:3 (v/v) (N. C. Kendrick, personal communication). A small quantity of arsenazo III was purified by paper chromatography for injection into photoreceptors (see Fig. 4*B*); however, except for Fig. 4*B*, all the data shown in this paper were obtained using acid-precipitated dye that contained minor amounts of impurities, the absorption spectra of which were insensitive to changes of $[Ca^{2+}]$.

Microspectrophotometry. Microspectrophotometry of single ventral photoreceptors was carried out using the modified Cary 14 (Varian, Palo Alto, Ca.) recording spectrophotometer described by P. K. Brown (1961, 1972). The present instrument uses a tungsten iodide light source (50 W, 12 V; Osram, Munich) and a photomultiplier tube (C31025C Quantacon; RCA, Harrison, N.J.) as detector. Cells were scanned from 750–400 nm at 2·5 nm sec^{-1} with a beam diameter of 40 μm. The measuring beam of the instrument did not noticeably light-adapt the photoreceptors. During some experiments a photoreceptor was impaled with a micropipette while positioned in the measuring beam of the instrument. This allowed the recording of dye spectra before and after intracellular injection of various ions (Ca^{2+}, Mg^{2+} or $EGTA^{2-}$) and the measurement of receptor potentials between scans.

To allow estimation of $[Ca^{2+}]_i$ from microspectrophotometric measurements, a standard calibration curve for the changes of absorption of arsenazo III as a function of calcium concentration was constructed as follows. Equal volumes of solutions containing different calcium concentrations (prepared by serial dilution) were added to aliquots of a solution of 2 mM arsenazo III in 400 mM-KCl, 25 mM MOPS (3-(*N*-morpholino) propane sulphonic acid at pH 7·0 and 1 mM-$MgCl_2$. The absorption spectra were measured in the microspectrophotometer using microcells with a 100 μm path length ('Microslides', Vitro Dynamics, Rockaway, N.J.). In Fig. 2, the absorption at 660 nm divided by the absorption at 585 nm (the isosbestic point) is plotted as a function of the total calcium concentration.

Similarly, to allow estimation of changes of intracellular pH detected by phenol red, a calibration curve for the changes in the absorption spectrum of phenol red as a function of pH was constructed. The pH of aliquots of a solution of 1 mM phenol red, 400 mM-KCl and 25 mM MOPS was adjusted by the addition of equal volumes of different concentrations of KOH. The absorption spectra were measured in the

microspectrophotometer using microcells with a 100 μm path length. The absorbance at 429 nm divided by that at 558 nm was plotted as a function of pH.

Microphotometry. Microphotometric measurements were made simultaneously with recordings of membrane voltage (or voltage-clamp current). White light from a 100 W 12 V tungsten-iodide lamp was passed through a ventral eye and focused by a microscope objective on to an iris diaphragm. This diaphragm restricted the measured light to that passing through a single cell; the light then was passed through an interference filter on to a photodetector, either a silicon photodiode operated in the unbiased mode (PV 100, E, G & G, Salem, Ma.) or a photomultiplier tube (R777, Hamamatsu, Middlesex, N.J.). The interference filters had centre wave-lengths of 660 or 670 nm (1/2 BP of 10 nm) or 585 nm (1/2 BP of 1·5 nm).

Stimulus light was calibrated by measuring the light falling on a 200 μm diameter pinhole placed at the locus of a photoreceptor cell, using a silicon photodiode photometer (J16, Tektronix, Beaverton, Or.).

In this paper, absorbance at a specific wave-length, λ nm, is denoted by A_λ; light transmission at that wave-length by T_λ.

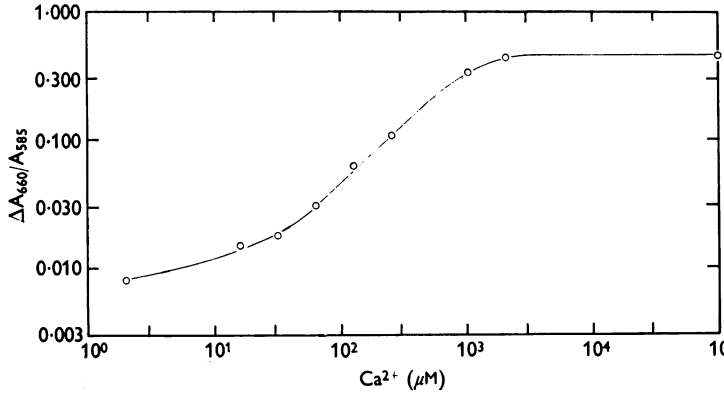

Fig. 2. Calibration curve for Ca^{2+}–arsenazo III complex formation. The change in absorbance at 660 nm normalized by the total absorbance at the isosbestic wave-length, 585 nm, is plotted as a function of calcium added. Arsenazo III (2 mM) in a solution of 400 nM-KCl, 25 mM MOPS at pH 7·0 and 1 mM-$MgCl_2$. Equal volumes of serially diluted $CaCl_2$ were added to aliquots of the dye solution and measured in microcuvettes (100 μm path length) in the microspectrophotometer.

RESULTS

Scanning microspectrophotometry

Single *Limulus* ventral photoreceptors were examined in the microspectrophotometer. A typical spectrum recorded from an uninjected cell is shown in Fig. 3. The apparent increase in absorbance at short wave-lengths in this spectrum is largely the consequence of the increase in light scattering at these wave-lengths; in this spectrum, there is no significant

absorption due to visual pigment. When measured under appropriate conditions, the visual pigment in these cells is known to absorb maximally at about 520 nm (Murray, 1966; P. K. Brown and J. E. Lisman, unpublished). Single photoreceptors were injected with arsenazo III and the micropipette was withdrawn. Absorption due to intracellular dye was

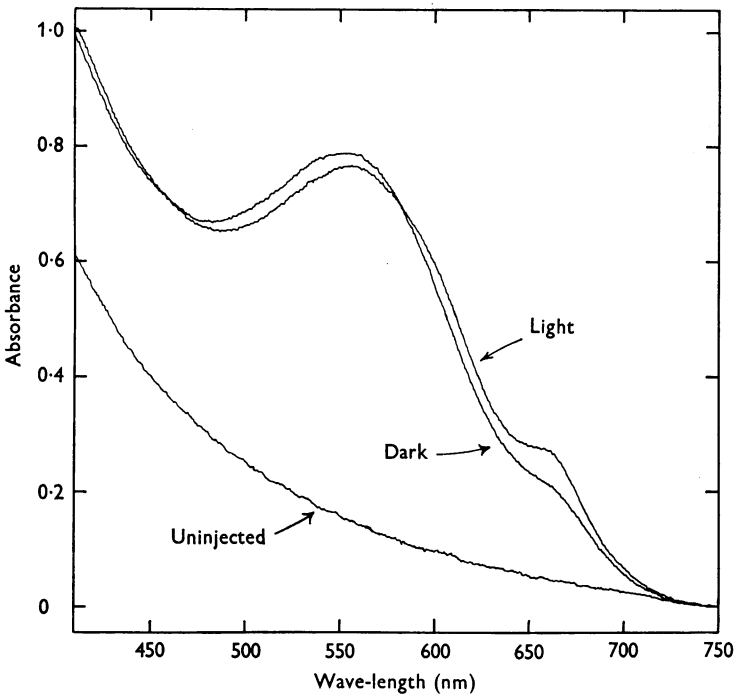

Fig. 3. Absorption spectra recorded microspectrophotometrically from single *Limulus* ventral photoreceptor cells. The spectrum from a cell (no. 10 of Table 1) injected with arsenazo III was first recorded after the cell was allowed to dark adapt for 15 min (labelled 'dark'). The cell was then illuminated ($8{\cdot}9 \times 10^{-3}$ W/cm^2 for 15 sec) and the spectrum again recorded (labelled 'light'). The spectrum labelled 'uninjected' was recorded from a neighbouring cell not injected with dye.

determined as the difference between the spectrum recorded from an injected cell and the typical spectrum recorded from a neighbouring uninjected cell. The dye concentration was monitored by dye absorption at 585 nm, the isosbestic wave-length for Ca^{2+}–arsenazo III binding. Absorption at 585 nm due to intracellular dye usually declined slowly (as much as 5 % per hour) during the course of microspectrophotometric observations. This decline in dye concentration was probably due to diffusion of dye into the axon of the photoreceptor, since dye could often be seen in the axon.

To compare spectra recorded at different times, the spectra were normalized to have the same value of A_{585}. Occasionally, a large decline of A_{585} was found during the course of an experiment; data from such a cell were discarded. A typical spectrum recorded from a partially dark-adapted cell injected with arsenazo III is shown in Fig. 3. The spectrum has a peak at about 550 nm due to absorption by the intracellular dye. After the cell was light-adapted, absorbance between about 450 and 585 nm decreased, whereas absorbance between 585 nm and about 740 nm became larger. As the cell was allowed to dark-adapt again, the spectrum changed back towards the initial spectrum. Since light-adaptation produces no significant change in the spectrum of an uninjected cell, the differences in the spectra recorded from a single cell before and after light-adaptation are due to the changes in absorption by the intracellular dye. To compare and normalize spectroscopic changes (cf. Fig. 3) measured in different cells, the difference in absorbance was measured, divided by the absorbance at the isosbestic point, A_{585}, and plotted. Such difference spectra for three cells are shown in Fig. 4*A*. These light-adapted minus dark-adapted difference spectra have a broad minimum at about 530 nm, an isosbestic point at about 585 nm and two maxima at about 610 and 660 nm. The amplitudes of the light-elicited difference spectra declined to zero as the cells were allowed to dark-adapt for 30 min or longer.

The rate at which the difference spectra declined towards zero as the cells dark-adapted influenced the shape of the spectra. For some cells the total duration of a scan (3 min) was an appreciable fraction of the time required for the difference spectra to approach zero. For such cells, if the absorption spectra are measured by scanning wave-lengths from 750 nm towards 450 nm, the difference spectra elicited by light-adaptation are relatively larger at longer wave-lengths than if the scans are made from 450 nm towards 750 nm (Fig. 4*B*). Therefore, the exact shapes of the difference spectra measured from intracellular dye cannot be compared with those measured in cuvettes.

Ionic specificity of spectroscopic changes

Difference spectra such as those shown in Fig. 4*A* are characteristic of arsenazo III–Ca^{2+} complex formation (see for comparison, Fig. 1*A*). These difference spectra were therefore compared with those obtained by changing intracellular ionized calcium in three ways. Firstly, intracellular ionized calcium was raised directly by iontophoretic injection (Lisman & Brown, 1972*a*, *b*; Brown & Lisman, 1975) into a dark-adapted cell previously injected with arsenazo III. The difference spectrum determined at the end of such a Ca^{2+} injection is shown in Fig. 4*C*. About 30 min after the Ca^{2+} injection, the spectrum recovered to that originally

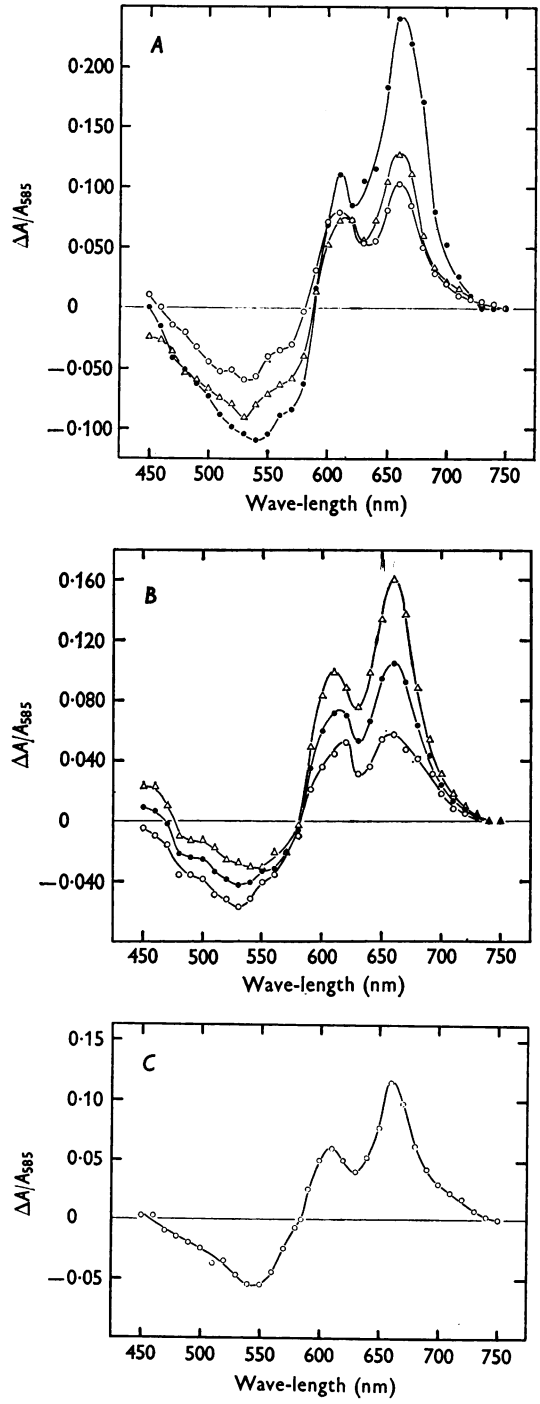

Fig. 4. For legend see facing page.

measured from the dark-adapted cell. The differences between dye spectra measured before and after direct intracellular injection of Ca^{2+} are similar to the differences elicited by light-adaptation.

Second, EGTA was injected iontophoretically into cells previously injected with arsenazo III. The spectrum measured from a light-adapted cell before EGTA injection subtracted from the spectrum measured from the same (light-adapted) cell after EGTA injection is shown in Fig. 5*A*. The injection of EGTA reversed the changes in the absorption spectrum that light-adaptation had elicited.

Third, the intracellular ionized calcium concentration was changed by changing the concentration of calcium in the sea water bathing a light-adapted cell. To do this, the spectrum was recorded from a dye injected cell that had been light-adapted while bathed in 10 mM-Ca^{2+} sea water. The chamber then was removed from the instrument and the solution replaced five successive times with 50 mM-Ca^{2+} sea water. The chamber was returned to the instrument and the cell re-aligned in the measuring beam. The cell was again light-adapted and the spectrum was recorded. A difference spectrum obtained by this procedure is shown in Fig. 5*B*. After raising extracellular Ca^{2+}, the change in absorption spectrum of the intracellular arsenazo III resembles that seen after intracellular iontophoretic injection of Ca^{2+}.

The changes in the absorption spectrum of intracellular arsenazo III were also examined after increasing intracellular Mg^{2+} directly by iontophoretic injection. Cells previously injected with dye were impaled with a micropipette containing 1·0 M-$MgCl_2$ and the difference spectra elicited by passing positive current out of the electrode were determined; such a difference spectrum (Fig. 6*A*) has a single maximum at about 620 nm and does not resemble the difference spectrum elicited by light-adaptation.

Fig. 4. *A*, light-adapted minus dark-adapted difference spectra, recorded from three cells injected with arsenazo III. The data from Fig. 3 are plotted as open circles. Cell no. 12 (△)and cell no. 1 (●) of Table 1 are also plotted. *B*, difference spectra measured from a single cell injected with arsenazo III that had been purified chromatographically. The light-adapted minus dark-adapted difference spectrum was determined by scanning from 750 to 450 nm (△) and also by scanning in reverse, from 450 to 750 nm (○). The average of those two scans is also plotted (●). $A_{585} = 0{\cdot}387$. *C*, difference spectrum due to direct iontophoretic injection of Ca^{2+} (5 nA for 120 sec out of the Ca^{2+}-containing micropipette) into a dark-adapted cell previously injected with arsenazo III. $A_{585} = 0{\cdot}201$.

Changes of intracellular pH

The absorption spectrum of arsenazo III changes with pH (cf. Fig. 1*C*). Therefore, we examined the possibility that light-induced spectroscopic changes such as those shown in Fig. 4*A* could be due to changes of intracellular pH. There are two reasons why this might happen. Firstly, because bright illumination apparently can lead to a rise of $[Ca^{2+}]_i$ (Brown & Blinks, 1974), a concomitant decline of intracellular pH might be expected,

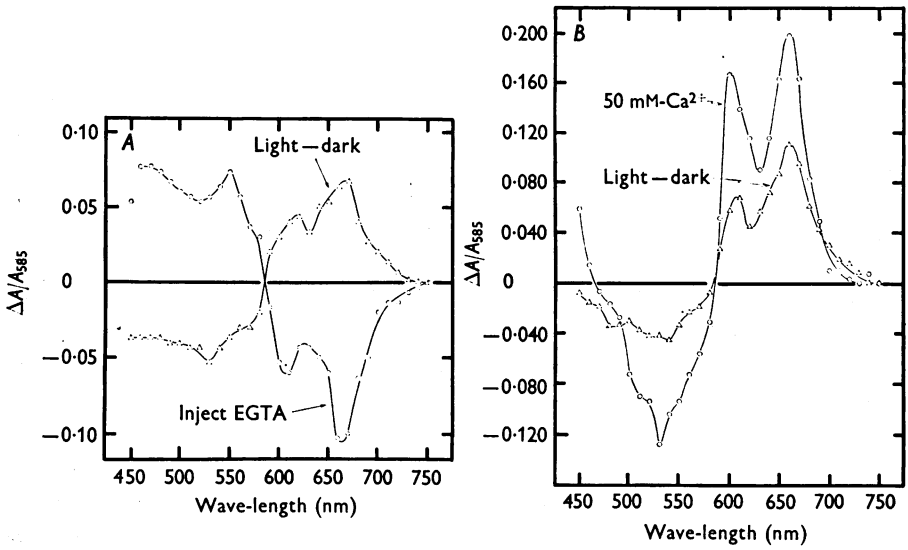

Fig. 5. Difference spectra recorded from single photoreceptors injected with arsenazo III. For each cell, the light-adapted minus dark-adapted difference spectrum is shown for comparison. *A*, the spectrum measured before injection was subtracted from the spectrum measured after intracellular iontophoretic injection of EGTA (4 nA for 10 min). The cell (no. 6 of Table 1) was light-adapted before each scan. *B*, the spectrum measured while the cell (no. 2 of Table 1) was bathed in 10 mM-Ca^{2+} sea water was subtracted from the spectrum measured while the cell was bathed in 50 mM-Ca^{2+} sea water.

since Ca^{2+} sequestration by mitochondria is known to be accompanied by proton extrusion (Lehninger, Carafoli & Rossi, 1967). Secondly, a recent report (Brown, Meech & Thomas, 1976) states that in barnacle photoreceptors, prolonged illumination leads to a small decrease of intracellular pH ($\sim 0{\cdot}1$ pH unit) as detected by pH sensitive micro-electrodes. In *Limulus* ventral photoreceptors, light-adaptation produced by a bright prolonged illumination (such as that used in the experiment illustrated in Fig. 3) elicits a small decrease of intracellular pH ($< 0{\cdot}1$ pH unit) as

detected by the changes in absorption of phenol red, a pH indicator dye, injected intracellularly (J. E. Brown and P. K. Brown, unpublished).

Changes in the absorption spectrum of intracellular arsenazo III therefore were measured after the intracellular pH was decreased by addition to the bath of some sea water saturated with CO_2. The change in intracellular pH in the ventral photoreceptors was monitored as follows. A neighbouring photoreceptor cell was injected with phenol red. The absorption due to intracellular phenol red was determined as the difference

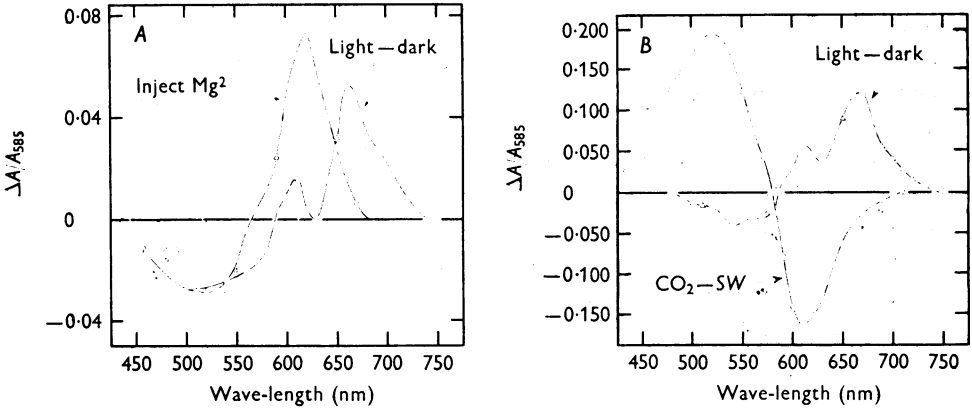

Fig. 6. Difference spectra of photoreceptors injected with arsenazo III. For each cell, the light-adapted minus dark-adapted difference spectrum is shown. *A*, the absorption spectrum measured after intracellular iontophoretic injection of Mg^{2+} (+10 nA for 3 min) was subtracted from the absorption spectrum measured before the Mg^{2+} injection; the cell (no. 4 of Table 1) was dark-adapted throughout. *B*, the absorption spectrum after the addition of an aliquot of sea water (*SW*) saturated with CO_2 was subtracted from the spectrum measured before addition of CO_2; the cell (no. 5 of Table 1) was dark-adapted throughout.

between the absorption spectrum of the cell containing dye and a typical spectrum measured from a neighbouring uninjected cell. The absorption spectrum of the intracellular phenol red was measured before and after addition of the sea water containing CO_2. From these spectra, the ratios of absorbances at 429 and 558 nm were determined and the change in intracellular pH estimated by means of a standard calibration curve. The addition of CO_2 in the experiment illustrated in Fig. 6*B* caused a decrease of 0·4 pH units as measured in the cell injected with phenol red. The difference spectrum of arsenazo III (shown in Fig. 6*B*) measured inside a neighbouring cell has a minimum at about 610 nm and is very different from the difference spectrum elicited by light-adaptation.

Microphotometry at single wave-lengths

Because the microspectrophotometer scanned slowly with respect to the kinetics of the change of $[Ca^{2+}]_i$ previously found by the aequorin technique, we attempted to detect rapidly the light-induced changes of $[Ca^{2+}]_i$ by measuring changes in absorption of arsenazo III at a single wave-length. To do this, *Limulus* ventral photoreceptors were impaled with micropipettes filled with arsenazo III. Before injection of the dye, a cell that had been dark-adapted for 5 min or longer responded to a bright and prolonged stimulus of white light with a depolarizing receptor potential having a transient phase that rapidly declined to a plateau. During the stimulus there was no change in the intensity of light that passed through the cell and reached the photodetector at 660 (or 670) nm. After small injections of arsenazo III, a bright prolonged stimulus presented to a dark-adapted cell still elicited a receptor potential having transient-to-plateau wave shape. The cells did not look stained when examined through the microscope by eye; however, during the stimulus, there was a rapid transient decrease in light transmission at 670 nm (T_{670}) followed by a slower recovery of T_{670} towards its initial value (Fig. 7*A* and *B*). After large injections of dye, cells looked markedly stained; a bright, prolonged stimulus presented to such a cell that had been dark-adapted for 5 min or longer elicited a 'square' receptor potential and a slow, progressive decrease in T_{670} (Fig. 7*B*). T_{670} took up to 30 min to recover in the dark.

The light-induced decrease in dye transmission at 660 nm occurred in cells voltage clamped to resting voltage and therefore did not depend on a change in membrane voltage. The change in T_{660} became undetectable in a cell previously injected with arsenazo III when EGTA was iontophoretically injected in sufficient amount to make the receptor potential square (Lisman & Brown, 1975).

The magnitude of the light-induced decrease of T_{660} depended on extracellular calcium concentration, being larger when the cells were bathed in 50 mM-Ca^{2+} sea water and decreasing when the sea water in the extracellular bath contained no Ca^{2+}. In addition, light induced an increase of transmission at 540 nm in a dye-injected cell. Light-induced changes in T_{660} were recorded also from cells injected with a solution containing 30 mM arsenazo III and 500 mM MOPS buffer, which therefore contained seventeen times more pH buffer than dye. All these findings suggest that the light-induced change in a cell injected with arsenazo III monitors a change in the intracellular concentration of ionized calcium.

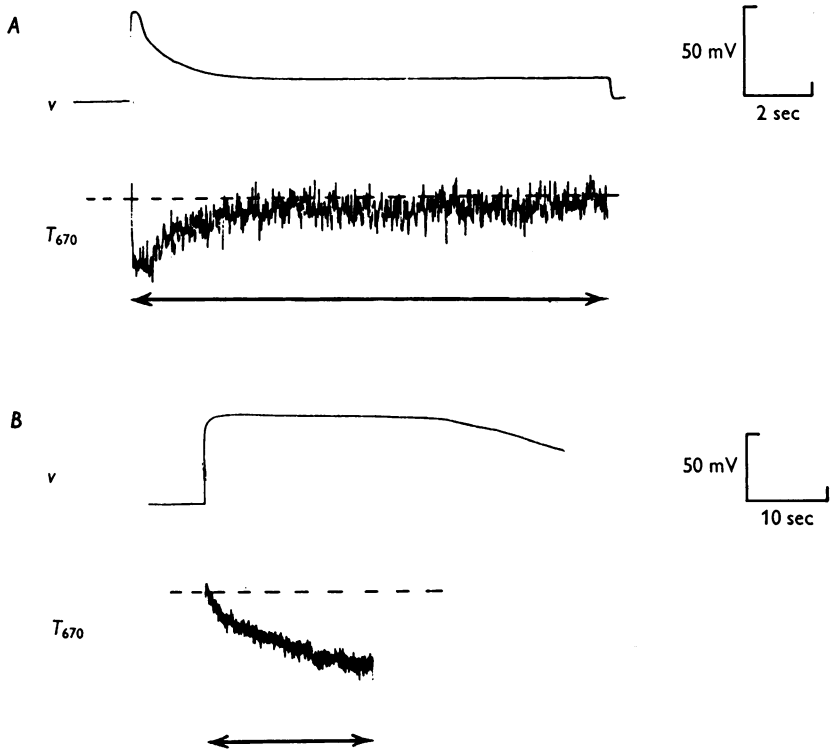

Fig. 7. Simultaneous recordings of light transmission at 670 nm (T_{670}) and membrane voltage in *Limulus* ventral photoreceptor, injected with arsenazo III. Membrane voltage was recorded with the same micropipette used for the injection of dye. The duration of the stimulus is indicated by double-ended arrows. T_{670}, recorded using a silicon photodiode, is shown with arbitrary scale. The dashed lines indicate the initial value of T_{670} when the stimulus was turned on. *A*, after a small injection of dye, the cell was allowed to dark-adapt for 10 min. A bright stimulus ($6{\cdot}0 \times 10^{-3}$ W/cm^2) elicited a transient decrease of T_{670}. *B*, after a large injection of dye the cell appeared markedly coloured. A bright, prolonged stimulus ($6{\cdot}0 \times 10^{-3}$ W/cm^2) elicited a prolonged decrease in T_{670} and the receptor potential was 'square.'

Concentration of intracellular dye

The changes in wave shape of the receptor potential after injection of arsenazo III depended on the amount of dye injected. After successively larger amounts of dye were injected, the decline from transient to plateau became slower and smaller (Fig. 8*B* and *C*); simultaneously, the kinetics of the decrease in T_{660} became slower.

To assess the effects of dye concentration more precisely, the volume of

dye solution injected into each of eight cells was measured by inclusion of radioactive sulphate in the injection solution. The volume of each cell was estimated by assuming that it was an ellipse of revolution with major and minor axes as measured by an eyepiece micrometer. The intracellular dye concentrations estimated by this procedure ranged between 0·7 and 6·8 mM. For dye concentrations below about 3·0 mM, there was a rapid decrease in T_{660} during a prolonged bright stimulus and the receptor potential had a rapid decline from transient to plateau (see inset in Fig. 9). For the larger dye concentrations, the kinetics of both the decline of the transient phase of the receptor potential and the transient in the dye response were slower.

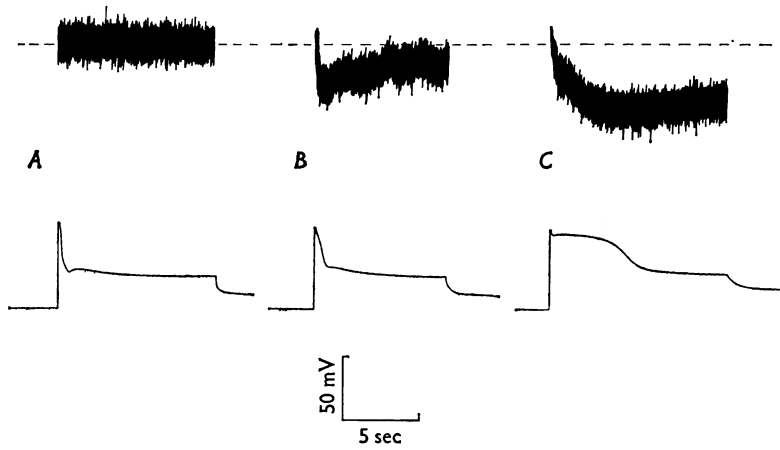

Fig. 8. Changes of T_{660} recorded from a single photoreceptor injected with arsenazo III as in Fig. 7. *A*, before injection; *B*, after a small injection; *C*, after an additional, larger injection of dye. Notice that after the injection of dye, both the decline of the transient phase of the receptor potential and the transient decrease of T_{660} became slower. T_{660} is shown with arbitrary scale. The stimulus intensity was $1{\cdot}3 \times 10^{-2}$ W/cm^2.

To allow estimation of the dye concentration in cells injected with dye but not with radioactive tracer, the graph in Fig. 9 was constructed by plotting the measured dye absorbance at 585 nm divided by the path-length (estimated as the minor axis of the cell outline) against the estimated dye concentration calculated from the known injection volume. The two measures of dye concentration are related as shown in the Figure; the slope of the line through the origin (fit by least-squares) is $7{\cdot}56 \times 10^3$ A_{585} cm^{-1}.M^{-1}. By referring to the graph in Fig. 9, the dye concentrations in the cells examined in the scanning microspectrophotometer ranged from 3·2 to 10·4 mM (see Table 1). These estimated concentrations

of intracellular arsenazo III assume that the dye injected was pure; as this was found by paper chromatography not to be so, the values will be slightly over-estimated.

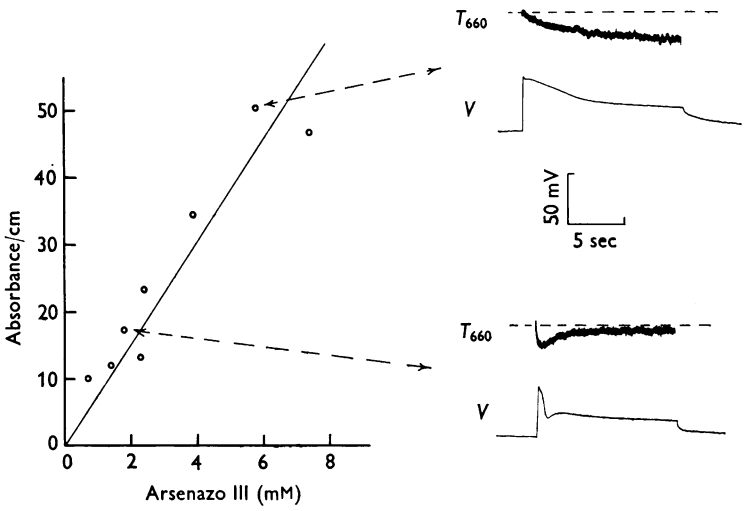

Fig. 9. The calculated absorbance per centimetre at 585 nm due to the injection of arsenazo III is plotted against the calculated intracellular concentration of the dye for eight cells. The volumes of dye solution injected were measured for each cell using a $^{35}SO_4$ tracer (see text). The insets show the wave forms of both T_{660} and membrane voltage (V) for the two cells indicated by dashed arrows. Notice that for the higher dye concentration, both the decline of the transient phase of the receptor potential and the decrease in T_{660} became slower. For both insets, the stimulus intensity was $1{\cdot}3 \times 10^{-2}$ W/cm^2.

DISCUSSION

Ionic basis of light-induced changes in the absorption spectrum of arsenazo III

The microspectrophotometric measurements from *Limulus* ventral photoreceptors injected intracellularly with arsenazo III have indicated the ionic basis of the changes in dye absorption following light-adaptation. The light-adapted minus dark-adapted difference spectra closely resemble the difference spectra induced by direct intracellular injection of Ca^{2+} ions but differ markedly from the difference spectra induced by direct intracellular injection of Mg^{2+} ions. Moreover, intracellular injection of EGTA reverses the light-induced changes of the arsenazo III spectrum. Since EGTA binds Ca^{2+} much more strongly than it does Mg^{2+}, this finding also indicates that the light-induced changes in the absorption spectrum of the

TABLE 1

Cell no.	A_{585}	White light stimulus		$\left(\frac{\Delta A_{660}}{A_{585}}\right)$	Estimated light-adapted $[Ca^{2+}]_i$ (10^{-4} M)	Estimated arsenazo III (mM)
		10^{-3} W/cm^2	Sec			
1	0·191	8·9	30	0·241	6·5	3·2
2	0·263	5·8	15	0·110	2·4	4·4
3	0·270	5·8	15	0·070	1·4	4·5
4	0·280	5·8	15	0·049	1·0	4·6
5	0·304	5·8	15	0·132	3·0	5·0
6	0·308	5·8	15	0·065	1·4	5·1
7	0·330	0·8	15	0·060	1·2	5·4
8	0·329	0·23	60	0·120	2·5	5·4
9	0·357	18·0	30	0·090	1·9	5·9
10	0·520	8·9	15	0·102	2·2	9·6
11	0·563	5·8	15	0·067	1·4	9·3
12	0·628	8·9	30	0·127	2·6	10·4

intracellular dye are due to binding of arsenazo III with calcium rather than with magnesium. In addition, changes of intracellular pH of the size and sign expected to occur normally after illumination do not produce spectroscopic changes resembling those induced by light-adaptation. Decreases of intracellular pH lead to changes of arsenazo III absorption that are opposite in sign to those seen in the light-adapted minus dark-adapted difference spectra. Furthermore, the difference spectrum induced by a change of pH does not have the characteristic double maxima at 610 and 660 nm that are induced either by light-adaptation or Ca^{2+} injection. Thus, it is very unlikely that the changes in arsenazo III absorption produced by light-adaptation are due to changes of intracellular pH. We conclude that the changes in the absorbance of intracellular arsenazo III indicate that there is a rise in intracellular ionized calcium concentration when a ventral photoreceptor is light-adapted.

$[Ca^{2+}]_i$ *of light-adapted photoreceptors*

The concentration of ionized Ca^{2+} in light-adapted ventral photoreceptors can be estimated from difference spectra such as those in Fig. 3 and the calibration curve in Fig. 2. The values of $[Ca^{2+}]_i$ for several cells are given in Table 1; these cells were injected with various concentrations of dye (all exceeding the dye concentration used to construct the calibration curve) and could not be brought to the same state of adaptation before stimulation. These factors, as well as the illuminance and duration of the adapting light, would be expected to influence the values of $[Ca^{2+}]_i$ obtained. Nevertheless, the estimated values of $[Ca^{2+}]_i$ ranged from 1·0 to

$6{\cdot}5 \times 10^{-4}$ M. These high values may indicate that some of the cells may have had large enough injections of solutions to cause $[Ca^{2+}]_i$ to be maintained at a high value; this phenomenon was previously observed after aequorin injection (Brown & Blinks, 1974). Also, injections of volumes greater than 10^{-10} l. (approximately 20% of the volume of the largest photoreceptor cells; Clark, Millecchia & Mauro, 1969) of any substance have been found to reduce the sensitivity to light and to slow the kinetics of the electrical responses (Lisman & Brown, 1975; see also Brown & Blinks, 1974; Coles & Brown, 1976); in the present experiments, such large injections raised the intracellular dye concentrations above 6 mM.

When the electrical responses of cells injected with less than 6 mM arsenazo III were examined during microspectrophotometric observation, they exhibited 'discrete events' (or 'quantum bumps') in the dark (Millecchia & Mauro, 1969) and a prominent transient phase of the receptor potential elicited by a bright, prolonged stimulus. In other words, their electrophysiology appeared to be normal. Therefore, the data in Table 1 suggest that $[Ca^{2+}]_i$ normally may reach a value of at least 10^{-4} M in a strongly light-adapted photoreceptor.

Kinetics of the changes of $[Ca^{2+}]_i$

The kinetics of the change of absorption at 660 nm, A_{660}, of intracellular arsenazo III depends on the concentration of the injected dye. With low concentrations (less than about 3 mM), A_{660} increases transiently, then declines to a value slightly greater than, or equal to, the value measured instantaneously at the beginning of the prolonged stimulus. The peak of the transient increase of A_{660} is delayed with respect to the peak of the electrical response of the cell to illumination. Larger injections of dye lead to slower kinetics of both the transient increase of A_{660} and the transient phase of the electrical response of the photoreceptor; with very large injections of the dye, the receptor potential becomes 'square'. These effects qualitatively resemble those seen after intracellular injection of EGTA. Lisman & Brown (1975) proposed that intracellular EGTA buffered changes of intracellular ionized calcium and tended to interrupt the reaction sequence whereby illumination leads to a reduction of sensitivity (i.e. light-adaptation). Since arsenazo III also binds calcium, a sufficiently large intracellular concentration of the dye might also buffer light-induced changes of $[Ca^{2+}]_i$ and tend to prevent desensitization. The monotonic increase of A_{660} seen in Fig. 7*B* supports this notion; the intracellular dye continued to bind $[Ca^{2+}]_i$ throughout the stimulus and no light-induced desensitization of the receptor potential became evident.

Ionic basis of rapid changes of A_{660}

The ionic basis for the rapid transient increase of A_{660} illustrated in Figs. 7*A* and 8*B* probably is the same as that inferred for the slower light-induced changes in absorption seen in the cells studied microspectrophotometrically. The two sets of phenomena have several common properties: both increase as extracellular calcium concentration is raised from 10 to 50 mM; both are abolished by intracellular injection of EGTA; and both have opposite signs at 540 and 660 nm. In addition, neither the rapid transient decreases in A_{660} measured after injection of small volumes of dye, nor the slower decrease in A_{660} measured in cells injected with large volumes of dye is noticeably changed if the cells are injected simultaneously with seventeen times more pH buffer than dye. From these observations we infer that the transient increases of A_{660} induced by light do not depend on changes of intracellular pH.

Moreover, the transient increase of T_{660} is unlikely to arise from an increase of $[Mg^{2+}]_i$. The selectivity of arsenazo III determined at 660 nm is at least thirty times greater for Ca^{2+} than Mg^{2+}. Therefore, light would have to induce an increase in $[Mg^{2+}]_i$ of at least 3 mM to produce the observed changes of A_{660}. However, the reversal voltage of the light-induced current is not changed by the removal of extracellular Mg^{2+} (Brown & Mote, 1974) and there is no other electrophysiological evidence for an influx of Mg^{2+} carrying a significant fraction of the light-induced current. To our knowledge, there is no mechanism that could account for increases of $[Mg^{2+}]_i$ as large as millimolar. Thus, it is unlikely that the light-induced change of A_{660} is caused by an increase of $[Mg^{2+}]_i$. We conclude that the rapid transient increase of A_{660} induced by illumination of a cell injected with less than 3 mM arsenazo III is caused by a light-induced transient increase of $[Ca^{2+}]_i$.

The initial rate of increase of A_{660} seen during prolonged illumination of a cell brought to a high concentration of arsenazo III was usually slower than the rate of rise seen with low concentrations (see Fig. 8 and insets of Fig. 9). The slower rate might occur for at least two reasons. Firstly, if the rate of change of A_{660} depends on that of the light response itself, the slower kinetics of the change of A_{660} might be due to the larger injection volume. As discussed above, large injections sometimes in themselves lead to desensitization and slower kinetics of the light response of ventral photoreceptors. Secondly, the kinetics of the receptor response itself depend upon $[Ca^{2+}]_i$; when $[Ca^{2+}]_i$ is raised, the time scale of the light response becomes faster (Brown & Lisman, 1975); conversely, when $[Ca^{2+}]_i$ is lowered, the time scale of the response becomes slower (Lisman & Brown, 1974; Srebro & Behbehani, 1975). Therefore, if

intracellular arsenazo III tended to buffer a light-induced increase of $[Ca^{2+}]_i$, the light response itself would be slower; this might account in part for the slower light-induced rise of $[Ca^{2+}]_i$ indicated by the dye.

In summary, when arsenazo III is injected intracellularly to low concentrations (< 3 mM), the rapid and transient increase of A_{660} of the intracellular dye indicates that light induces a transient rise of $[Ca^{2+}]_i$, confirming the findings by the aequorin technique (Brown & Blinks, 1974). A prolonged bright stimulus elicits a rise of $[Ca^{2+}]_i$ that reaches its maximum value after the peak of the transient phase of the electrical response, then falls to a lower level during the stimulus and returns to a still lower level when the cell is returned to darkness. These findings are consistent with the hypothesis that an increase of $[Ca^{2+}]_i$ is normally a step in the sequence of reactions of light-adaptation in *Limulus* ventral photoreceptors.

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REFERENCES

ASHLEY, C. C. (1970). An estimate of calcium concentration changes during the contraction of single muscle fibres. *J. Physiol.* **210**, 133–134*P*.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BLINKS, J. R. (1973). Calcium transients in striated muscle cells. *Eur. J. Cardiol.* **1**, 125–142.

BLINKS, J. R., PRENDERGAST, F. G. & ALLEN, D. G. (1976). Photoproteins as biological calcium indicators. *Pharmac. Rev.* **28**, 1–93.

BROWN, H. M., MEECH, R. & THOMAS, R. C. (1976). pH Changes induced by light in large *Balanus* photoreceptors. *Biophys. J.* **16**, 33a.

BROWN, J. E. & BLINKS, J. R. (1974). Changes in intracellular free calcium during illumination of invertebrate photoreceptors: detection with aequorin. *J. gen. Physiol.* **64**, 643–665.

BROWN, J. E., COHEN, L. B., DEWEER, P., PINTO, L. H., ROSS, W. N & SALZBURG, B. M. (1975). Rapid changes of intracellular free calcium concentration: detection by metallochromic indicator dyes in squid giant axon. *Biophys. J.* **15**, 1155–60.

BROWN, J. E. & LISMAN, J. E. (1975). Intracellular Ca modulates both sensitivity and time scale in *Limulus* ventral photoreceptors. *Nature, Lond.* **258**, 252–3.

BROWN, J. E. & MOTE, M. I. (1974). Ionic dependence of reversal voltage of the light response in *Limulus* ventral photoreceptors. *J. gen. Physiol.* **63**, 337–350.

BROWN, P. K. (1961). A system for microspectrophotometry employing a commercial recording spectrophotometer. *J. opt. Soc. Am.* **51**, 1000–1008.

BROWN, P. K. (1972). Rhodopsin rotates in the visual receptor membrane. *Nature, New Biol.* **236**, 35–38.

BUDESINSKY, B. (1969*a*). Acidity of several chromotropic acid azo derivatives. *Talanta* **16**, 1277–1288.

BUDESINSKY, B. (1969*b*). Monoarylazo and bis(arylazo) derivatives of chromotropic acid as photometric reagents. In *Chelates in Analytical Chemistry*, vol. 2, ed. FLASCHKA, H. A. & BARNARD, A. S., pp. 1–91. New York: M. Dekker.

CLARK, A. W., MILLECCHIA, R. & MAURO, A. (1969). The ventral photoreceptor cells of *Limulus*. I. The microanatomy. *J. gen. Physiol.* **54**, 289–309.

COLES, J. A. & BROWN, J. E. (1976). Effects of increased intracellular pH-buffering capacity on the light response of *Limulus* ventral photoreceptor. *Biochim. biophys. Acta* **436**, 140–153.

DEMOLL, R. (1914). Die Augen von *Limulus*. *Zool. Jb.* (*Anat.*) **38**, 443–464.

FEIN, A. & LISMAN, J. E. (1975). Localized desensitization of *Limulus* photoreceptors produced by light or intracellular Ca^{2+} injection. *Science, N.Y.* **187**, 1094–1096.

LEHNINGER, A. L., CARAFOLI, E. & ROSSI, C. S. (1967). Energy-linked ion movements in mitochondrial systems. *Adv. Enzymol.* **29**, 259–320.

LISMAN, J. E. & BROWN, J. E. (1971). Two light-induced processes in the photoreceptor cells in *Limulus* ventral eye. *J. gen. Physiol.* **58**, 544–567.

LISMAN, J. E. & BROWN, J. E. (1972*a*). The effects of intracellular iontophoretic injection of calcium and sodium ions on the light responses of *Limulus* ventral photoreceptors. In *The Visual System: Neurophysiology, Biophysics and their Clinical Applications*, ed. ARDEN, G. B., pp. 23–33. New York: Plenum.

LISMAN, J. E. & BROWN, J. E. (1972*b*). The effects of intracellular iontophoretic injection of sodium and calcium ions on the light response of *Limulus* ventral photoreceptors. *J. gen. Physiol.* **59**, 701–719.

LISMAN, J. E. & BROWN, J. E. (1974). Effects of calcium on adaptation and excitation in *Limulus* ventral photoreceptors. In *Assoc. Res. Vision Ophthal. Abstracts*, p. 13.

LISMAN, J. E. & BROWN, J. E. (1975). Effects of intracellular injection of calcium buffers on light adaptation in *Limulus* ventral photoreceptors. *J. gen. Physiol.* **66**, 489–506.

MICHAYLOVA, V. & ILKOVA, P. (1971). Photometric determination of micro amounts of calcium with arsenazo III. *Analytica chim. Acta* **53**, 194–198.

MICHAYLOVA, V. & KOULEVA, N. (1973). Arsenazo III as metallochromic indicator for complexometric determination of calcium in slightly alkaline medium. *Talanta* **20**, 453–458.

MILLECCHIA, R. & MAURO, A. (1969). The ventral photoreceptor cells of *Limulus*. II. The basic photoresponse. *J. gen. Physiol.* **54**, 310–330.

MURRAY, G. (1966). Intracellular absorption difference spectrum of *Limulus* extraocular photolabile pigment. *Science, N.Y.* **154**, 1182–1183.

REUBEN, J. P., BRANDT, P. W. & GRUNDFEST, H. (1974). Regulation of myoplasmic calcium concentration in intact crayfish muscle fibers. *J. Mechanochem. & cell. Motility* **2**, 269–285.

SAVVIN, S. F. (1961). Analytical use of arsenazo III. Determination of thorium, zirconium, uranium and rare earth elements. *Talanta* **8**, 673–685.

SAVVIN, S. B., AKIMOVA, T. G., KRYSIH, E. P. & DAVYDOVA, M. M. (1970). Electrophoretic and chromatographic methods of analyzing azo compounds. I. Analysis of arsenazo III and its analogs. *Zh. analit. Khim.* **25**, 430–433.

SREBRO, R. & BEHBEHANI, M. (1975). Calcium and the phototransduction process in the *Limulus* photoreceptor. *Biophys. J.* **15**, 169a.