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## DYE TRANSPORT ACROSS THE RETINAL BASEMENT MEMBRANE OF THE BLOWFLY *CALLIPHORA* *ERYTHROCEPHALA*

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### Summary

In the blowfly, *Calliphora erythrocephala*, transport of dye into or out of the retina, following injection into the eye or thorax, was investigated, mainly by microspectrophotometry and fluorimetry. After injection into the eye, Phenol Red, Trypan Blue, Lucifer Yellow and 9-amino-acridine were transported out of the retina; Procion Yellow and Rhodamine-123 stayed in it. The time constants of this transport process were in the range 45–80 min at 23°C, depending on the dye. When Lucifer Yellow was injected into the thorax, it was transported into the retina. The transport of these dyes across the retina was inhibited by nitrogen, ouabain and low temperature, indicating that active processes are involved.

### Introduction

The retina of insects shows some characteristics of a closed compartment. Often the composition of the eye haemolymph differs from that of the rest of the head, thorax and abdomen (see Treherne, 1985), and substances injected into the body show restricted entry into the retina (Shaw, 1977). The compartmentation is mediated by a diffusion barrier just proximal to the basement membrane (Shaw, 1984). Nevertheless, there must be some active uptake process for sugars, faster than diffusion, from the head haemolymph into the retina (Langer, 1962; Shaw, 1977). The disappearance of Lucifer Yellow was also noted by Wilcox & Franceschini (1984a) 24 h after injection into the retina.

The transport of substances into and out of the retina must play an important part in the maintenance of retinal function. As a preliminary to studies of the pharmacology of retinal processes (E. Weyrauther, J. G. H. Roebroek & D. G. Stavenga, in preparation) we have investigated such transport by studying the transport of various dyes.

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Key words: insect retina, dyes, transport, metabolism, blowflies.

## Materials and methods

### *Flies*

Blowflies *Calliphora erythrocephala*, mutant chalky, were reared on liver in the laboratory and used about 14 days after emergence. No differences in the results were found between males and females.

### *Dyes*

All chemicals used were from local suppliers. Phenol Red [3,3-bis(*p*-hydroxyphenyl)-2,1,3H-benzoxathiole 1,1 dioxide], Trypan Blue (sodium ditolyldisazobis-8-amino-1-naphthol-5,6-disulphonate), Lucifer Yellow CH and Procion Yellow M4-RAN were dissolved in insect Ringer's solution (composition in  $\text{mmol l}^{-1}$ : NaCl, 130; KCl, 2;  $\text{CaCl}_2$ , 2;  $\text{MgCl}_2$ , 5; Hepes, 10) at pH 7.2. 9-Amino-acridine was first dissolved in a small volume of ethanol, Rhodamine-123 [2-(6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid methyl ester] in a small volume of dimethylsulphoxide (DMSO), then diluted with Ringer's solution. For the quantitative measurements, either the strong absorption of Phenol Red (at neutral pH) in the green (peak at 560 nm) (Figs 2, 3, 7) or that of Trypan Blue (a single absorption peak at 588 nm) was used; in the fluorescence measurements of Lucifer Yellow, the excitation band was 390–490 nm and the emission above 515 nm was measured. Illumination of a drop of Lucifer Yellow solution on a coverglass with an intensity as used in the experiment shown in Fig. 4 induced fading out of less than 3% of the emission, even after more than 10 h.

### *Injection procedure*

Flies were immobilized in wax and mounted in a housing on a goniometer stage of the microscope (see below). Injections were made into the eye or the thorax by means of a glass microelectrode (tip diameter approx.  $30 \mu\text{m}$ ), using water pressure from a syringe. When injecting into the eye, a small hole was first cut in the cornea with a piece of razor blade. The volume of the solutions injected into the eye was about  $0.2 \mu\text{l}$ , and that injected into the thorax  $1\text{--}2 \mu\text{l}$ .

Damage to the retina or the thorax, or toxic effects of the dyes, could not be detected. The volume of  $0.2 \mu\text{l}$  injected into the eye is relatively large compared with the extracellular space ( $<0.1 \mu\text{l}$ ; Hamdorf *et al.* 1988). Although during injection a movement of the deep pseudopupil (Franceschini, 1975; Stavenga, 1979) could be observed in the direction away from the injection point, some minutes after injection it looked normal again. The volume injected in the thorax was about one-quarter of the total haemolymph volume (data for a fly comparable in size: *Calliphora vicina*:  $5\text{--}7 \mu\text{l}$ ; Jones, 1977). Some tests of the normal function of the eye after dye injections in the eye and thorax were made. No differences were found compared with untreated flies in pupillary response and mitochondrial activity, even after some hours.

*Photometric measurements*

The photometric measurements were made as described by Tinbergen & Stavenga (1986, 1987). The microspectrophotometer was a Leitz Orthoplan microscope equipped with a Ploemopak illuminator, a Compact photometer and a Leitz objective (NPL 10, 0.20). Epi-illumination was administered with xenon arc lamps. For absorption measurements, the retina was trans-illuminated from the ventral to dorsal side with light coming from a light guide (diameter 0.5 mm). The field diaphragm was focused on the dorsal cornea and light was collected from about 30 facets. An Oriel monochromator (bandwidth about 10 nm) was used for the spectral scans (500–650 nm, scan speed:  $10 \text{ nm s}^{-1}$ ) yielding the absorbance difference spectra. For the fluorescence measurements, the epi-illumination, blue fluorescence cube was used (excitation band 390–490 nm, emission band  $>515 \text{ nm}$ ). Incident light coming from the objective was focused at the level of the ‘deep pseudopupil’ (Franceschini & Kirschfeld, 1971). The diameter of the field diaphragm was the same as in the absorption measurements. Stimulus control and data acquisition were performed by a Data General MP 200 computer.

*Histology*

Histological procedures for Lucifer Yellow and Procion Yellow followed the methods given by Strausfeld *et al.* (1983) and Bishop (1980). The dyes were injected into intact flies, and after 1 or 2 h the flies were fixed, dehydrated in an ethanol series, and embedded in paraffin. Sections ( $10 \mu\text{m}$ ) were cut on a microtome (Bright Instrument Company Ltd), the embedding medium was dissolved out and sections were coverslipped with Fluoromount.

*Photographs*

The photographs were made either with a Leitz Orthomat camera mounted on the Orthoplan microscope or with a camera (Canon) on a binocular microscope. Artificial light diapositive film was used.

**Results***Injection of dyes into the retina*

Injection of a saturated Phenol Red solution of neutral pH in fly Ringer’s solution into the eye of a blowfly, mutant chalky, changed the normal white colour into a bright red (Fig. 1). After about 30 min the red colour had clearly lost its intensity, and about 2 or 3 h after injection the eye looked similar to the untreated one, except at the injection point, where some remnant of the red colour stayed. Evidently, the dye had vanished.

The phenomenon was investigated quantitatively by measuring the spectral transmission of the eye as a function of time. From these data, absorbance difference spectra were calculated (Fig. 2), which were (very approximately) proportional to the absorption spectrum of Phenol Red (Lisman & Strong, 1979).

The time course of the absorbance at the peak wavelength is shown in Fig. 3. The absorbance decrease closely follows an exponential given by  $y(t) = A \times \exp(-t/\tau)$ , with  $A = 0.77$  and  $\tau = 55$  min.

The inset of Fig. 3 shows the same experiment with another fly at a higher time resolution immediately after injection of the Phenol Red solution. In the first phase, lasting a few minutes, the absorption increased because the injection point was at the anterior side of the eye and the measuring light beam traversed mostly through posterior eye regions. This resulted in spreading out of the dye into the light path of the measuring beam. The second phase, in which the absorbance decreased, reflects the clearing of the dye from the retina. In all experiments made with Phenol Red, the time constant of the clearing process varied between 45 and 60 min (23°C). Control experiments (injection with Ringer's solution only) showed that the injection procedure itself did not result in any significant transmission or fluorescence change. Experiments with Trypan Blue yielded results identical to those obtained with Phenol Red (data not shown). Both dyes vanished from the retina at the same speed.

Slightly different observations were made with Lucifer Yellow. Local injection gave an initial rise of the emission in eye regions remote from the injection point, but with a slower time course than that of the initial absorbance increase which followed Phenol Red injection (compare Fig. 4 with Fig. 3, inset). Then the emission also dropped with an exponential time course, again slower than that in

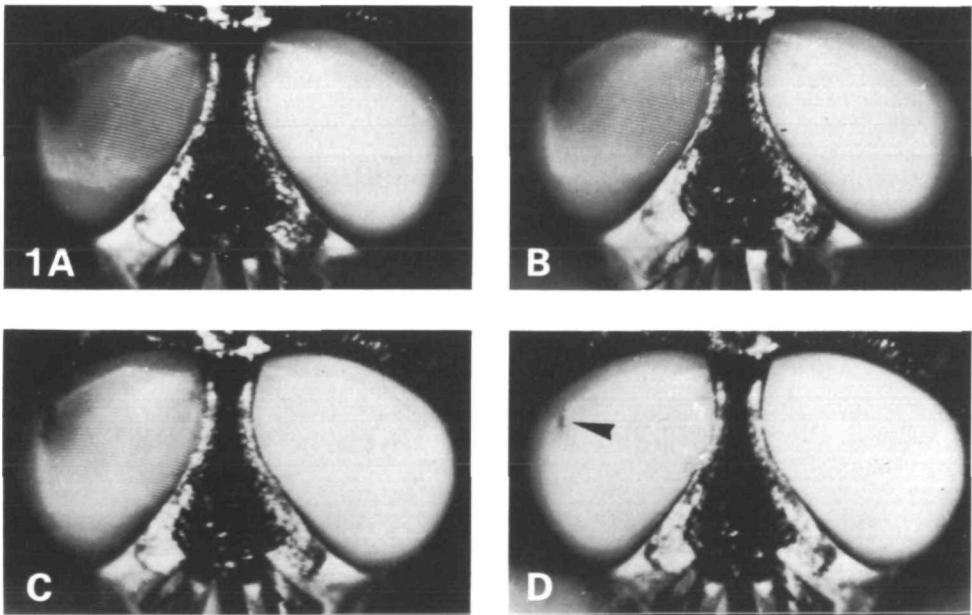


Fig. 1. Head of a male blowfly (*Calliphora erythrocephala*, mutant chalky) at different times after injection of Phenol Red into the right eye. The dark spot on the left side of the right eye is the injection point (arrowhead). Times after injection: A, 5 min; B, 30 min; C, 1 h; and D, 4 h.

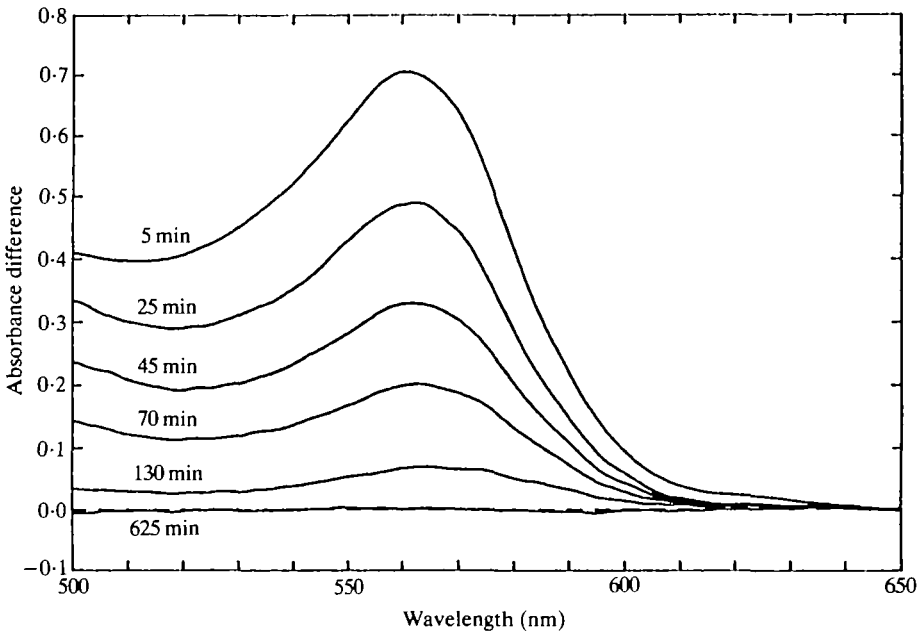


Fig. 2. Absorbance difference spectra of an eye of *Calliphora erythrocephala* calculated from transmission spectra measured at different times after injection of a Phenol Red solution into the eye. The reference was the transmission spectrum of the eye before injection.

the Phenol Red experiments. In the example of Fig. 4 the time constant of the exponential is 78 min. Furthermore, in contrast with the results from the nonfluorescing dyes, a steady emission level of about 40% of the maximum remained in the eye for at least several hours.

An experiment with 9-amino-acridine yielded a similar result: after injection and an initial emission increase the emission decreased, but with a slower time course than that observed with the nonfluorescing dyes.

No clear emission decrease occurred when Procion Yellow or Rhodamine-123 (Rh-123) was injected into the eye. Soon after injection, the emission increased due to the spreading of dye throughout the retina. The fluorescence subsequently increased slightly over about 5 h by 10%. Possibly this effect is due to very slow diffusion, but the increase is within the statistical error.

#### *Injection of dyes into the thorax*

The decrease of absorption by Phenol Red and of emission by Lucifer Yellow can, in principle, be caused by degradation or quenching. However, it is more likely to be the consequence of an exchange mechanism transporting the dyes out of the retina.

Lucifer Yellow was injected into the thorax and the emission of the eye was measured. The results varied with the injected concentration. Within several

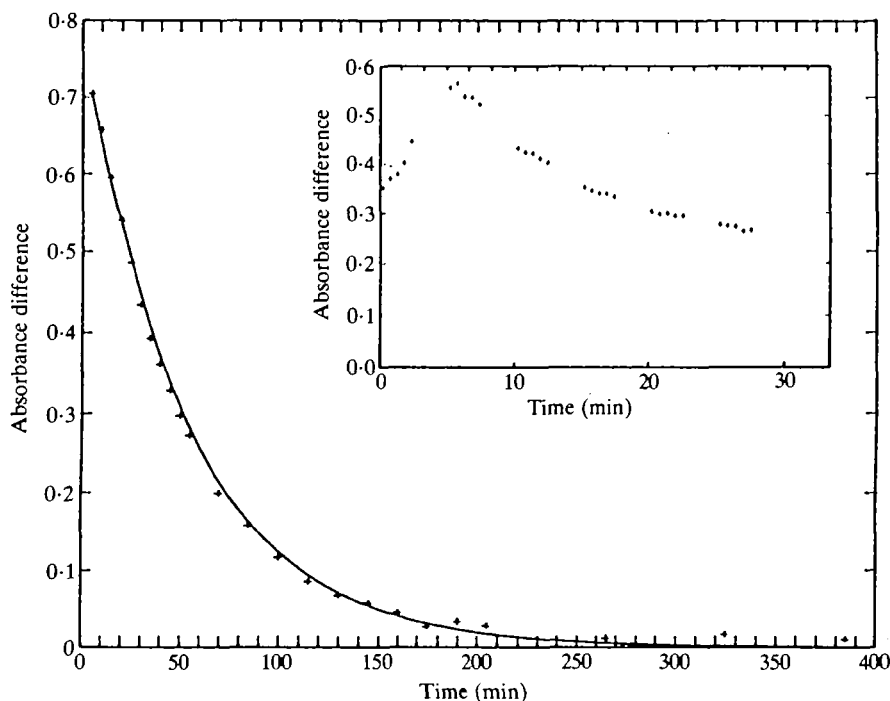


Fig. 3. Absorbance difference at 560 nm (the peak of Phenol Red absorption) depending on time. The solid line is the fitted exponential (least mean square) to the measured points (see text). The data are the same as in Fig. 2. Inset: the initial part of the same experiment on another fly with higher time resolution.

seconds after injection the fluorescence increased. This enhancement resulted from a very rapid transport of the injected dye solution into the head, as can be concluded from direct visual inspection. Immediately after injection a higher fluorescence could be seen, especially at the edges of the eye, where the layer of the eye distal to the head haemolymph is thinner than in the middle of the eye. Injection with a sufficiently high concentration of Lucifer Yellow into the thorax resulted in distinctly yellow-coloured eyes after some hours. The sequence is demonstrated in Fig. 5.

Quantitative measurements of the blue-induced yellow fluorescence are shown in Fig. 6. Injection of  $1 \text{ mmol l}^{-1}$  Lucifer Yellow resulted in a negligibly small increase in fluorescence directly after injection. Injection of 50 or  $200 \text{ mmol l}^{-1}$  Lucifer Yellow gave a noticeable, immediate increase and then fluorescence steadily increased up to a high level (Fig. 6). Procion Yellow and Rhodamine-123 injected into the thorax also resulted in a fluorescence increase directly after injection, but gave no further increase of emission with time.

Injections into the thorax of the nonfluorescing dyes were unsuccessful due to the low solubility of these dyes. Injection of a saturated solution, which is about

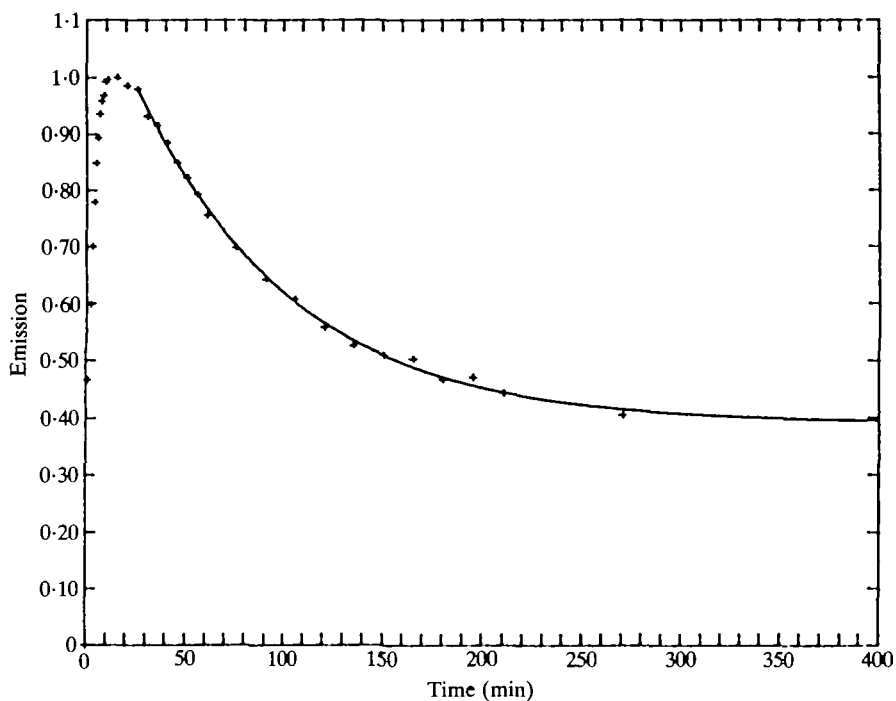


Fig. 4. The time course of emission ( $>515$  nm) of  $1 \text{ mmol}^{-1}$  Lucifer Yellow injected into an eye. The natural background emission of the eye is subtracted and the emission difference normalized. The solid line is the fitted exponential to the measured points (see text).

$2 \text{ mmol}^{-1}$  for Phenol Red and  $1 \text{ mmol}^{-1}$  for Trypan Blue, did not give a measurable change in absorption of the eyes.

### *Histology*

From histological sections of the eyes and bodies of flies after dye injection it was clear that Lucifer Yellow injected into the retina entered the body; when injected into the thorax it reached the head, and subsequently entered the retina. Procion Yellow, when injected into the retina, stayed there and, when injected into the thorax, reached the abdomen and parts of the head but did not appear to enter the retina.

### *Effects of inhibitors*

To investigate whether the transport of dyes into and out of the retina involved active transport, the following experiments were carried out. Nitrogen totally blocked the decrease of the Phenol Red absorption (Fig. 7). Pure nitrogen was blown around the fly for 5 min before injection and up to 15 min after it. During this time no decrease in absorption occurred (inset of Fig. 7). The nitrogen effectively anaesthetized the fly, as shown by the blockage of light-induced mitochondrial activity (Stavenga & Tinbergen, 1983), and apparently blocked the



transport system. After the nitrogen supply had been stopped, the fly recovered rapidly (within 1 min) from anaesthetization, and subsequently absorption decreased, although with a time constant somewhat longer than normal (Fig. 7). When  $5 \text{ mmol l}^{-1}$  ouabain was added to the Phenol Red solution the decay time constant increased to 100 min.

Temperature also affected the transport system, as expected. Whereas at  $23^\circ\text{C}$  the absorbance by Phenol Red 50 min after injection had fallen to about 40% of the initial value (Fig. 3), at  $13^\circ\text{C}$  only a 10% decrease occurred during this period. We did not attempt to determine accurately the temperature-dependence of the system, but clearly the process speeded up with an increase in temperature.

### Discussion

The experiments show that dyes introduced into the fly retina can reach the

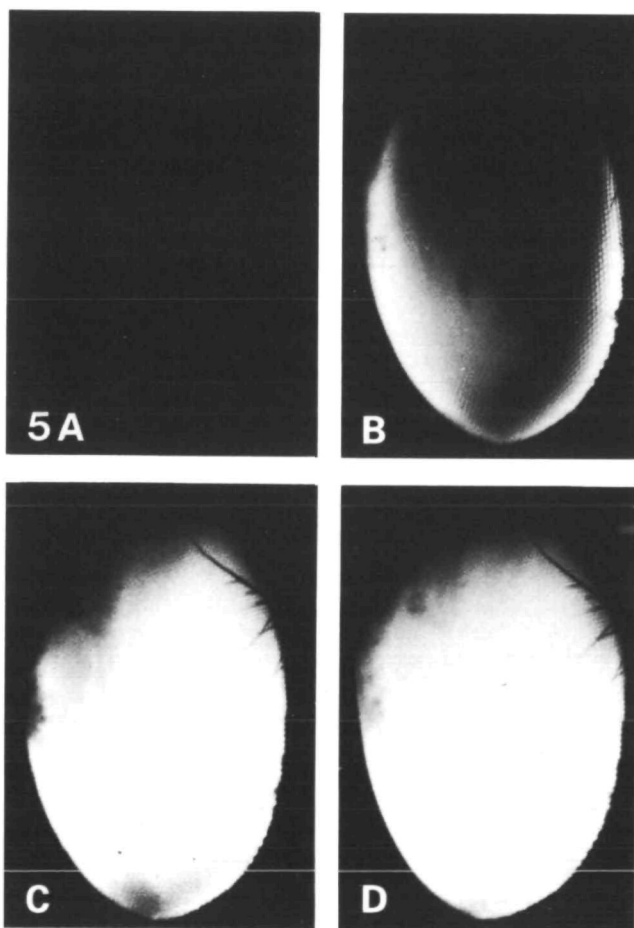


Fig. 5. Emission ( $>515 \text{ nm}$ ) from the right eye of a male fly before (A), and 5 min (B), 1 h (C) and 2 h (D) after injection of  $50 \text{ mmol l}^{-1}$  Lucifer Yellow into the thorax.

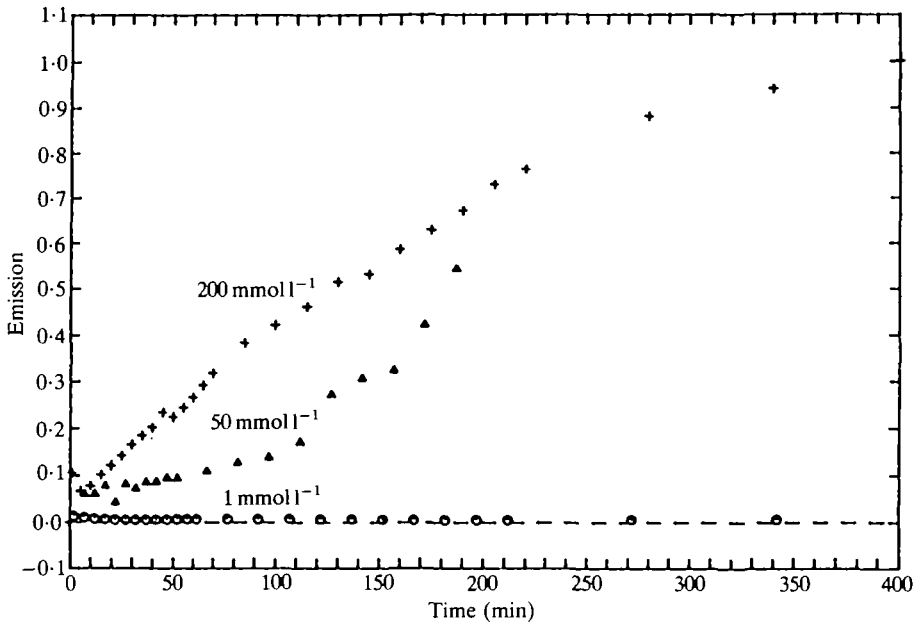


Fig. 6. The time course of the emission of blowfly eyes after injection of Lucifer Yellow solutions at various concentrations into the thorax. The natural background emission of the eyes is subtracted and the emission difference normalized to the emission of the  $200 \text{ mmol l}^{-1}$  experiment measured 400 min after injection.

body and *vice versa*. The transport system(s) carrying out this exchange must have a distinct biological function. For instance, the eye has to be supplied with nutrients from the midgut, and metabolic breakdown products have to reach the Malpighian tubules to be transported out of the insect's body. These transport phenomena seem to be faced with a serious constraint. The existence of a distinct barrier has often been demonstrated in ultrastructural (Chi & Carlson, 1981; Lane, 1981; Saint Marie & Carlson, 1983; Shaw, 1978), electrophysiological (Shaw, 1975; Zimmerman, 1978) and dye and tracer distribution studies (Lane, 1981; Shaw, 1977). The nature of the barrier has not been resolved, but its location must clearly be sought just proximal to the basement membrane (Shaw, 1984).

When a dye solution is injected into the eye of the blowfly, it spreads very rapidly, in seconds, throughout the whole eye (Wilcox & Franceschini, 1984*b*; el-Gammal *et al.* 1987). This spreading is not by diffusion or by active transport through the retina, but is due to the pressure of the injection. Still, even with the pressure-injection technique used here, the distribution of the dye in the retina is not uniform within a few minutes after injection (Figs 1, 3, 4).

Overlying the rapid spreading by injection and the slower diffusion in the extracellular space is a clearance of dyes from the retina, which is shown in Figs 1, 2 and 3 with Phenol Red. This clearing has an exponential time course (Fig. 3)

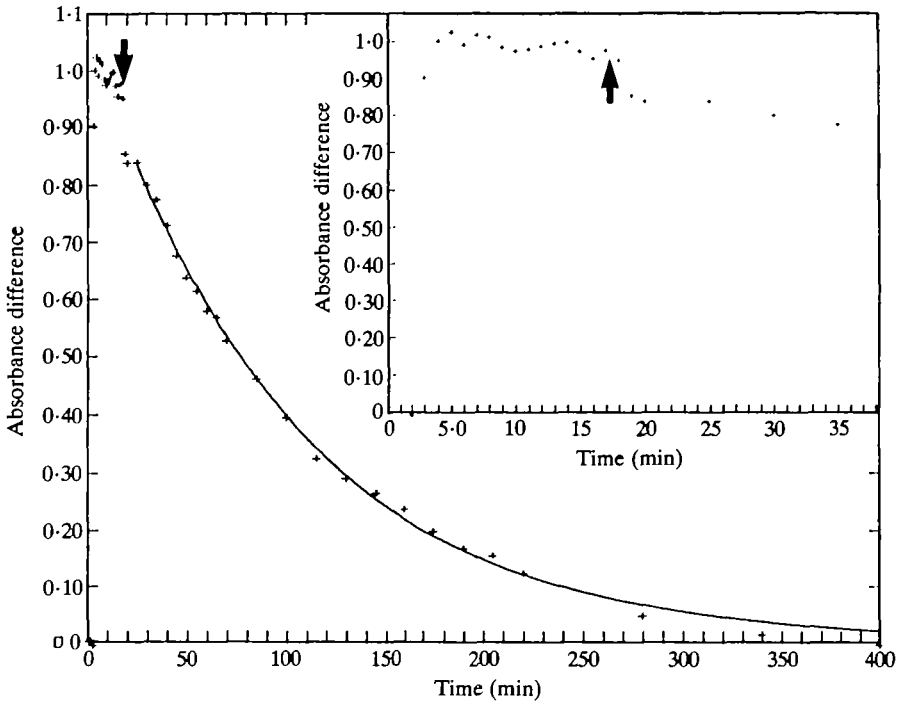


Fig. 7. The absorbance differences at 560 nm between the untreated eye and absorption at various times after the injection of a Phenol Red solution into the eye. The fly was anaesthetized with nitrogen 5 min before the start of the experiment. The injection was made between the points at 2 min and 2.5 min (see inset). Nitrogen flow was stopped 17 min after the start of the experiment (arrows). The solid line is the fitted exponential to the measured points from 25 to 340 min. Inset: the initial phase of the experiment shown with higher time resolution.

with a time constant of about 55 min (at 23°C), so that after 2 h less than 10 % of the injected dye still persists in the retina.

The decrease of Lucifer Yellow emission in the eye is slower (Fig. 4). The reason may be the loose binding of Lucifer Yellow to tissue structures (Strausfeld *et al.* 1983). This binding results in a slightly restricted diffusion and thus explains the slower increase of emission compared with the absorbance difference increase of Phenol Red (Fig. 3, inset). The slower disappearance of Lucifer Yellow fits with the results of Wilcox & Franceschini (1984a) on the housefly, who found that a light-induced uptake of Lucifer Yellow into the retinula cells is still possible 3 h after injection, but not after 24 h.

The mechanism causing the disappearance of the dyes could be degradation in the eye itself, diffusion, or active transport out of the retina. If the mechanism is transport of substances out of the retina, then there should be a transport route into the retina, too. The necessary existence of such a route, at least for carbohydrates, the most important energy supply in insects, has been shown by Langer (1962) and Shaw (1977). The latter author, working on locusts, dis-

tinguished between: (i) sulphate, sucrose, sorbitol and mannitol, which were taken up rapidly by an active process by glia or receptor cells, (ii) inulin and polyethylene glycol, whose uptake into the retina is much retarded compared with free diffusion, and (iii) Procion Yellow, which did not enter the eye at all.

In blowflies Lucifer Yellow, Procion Yellow and Rh-123, when injected into the thorax, all enter the head very rapidly and reach the area below the eyes (Fig. 5). This rapid transport is probably mediated by the circulation of the haemolymph driven by the heart. Jones (1977) showed for the cockroach that a dye injected in the abdomen appears in the head after 30 s. After the heart stops pumping owing to some external upset, the first beats when it restarts are generally in the direction of the head (L. T. Wasserthal, personal communication). Only Lucifer Yellow enters the retina, but Procion Yellow and Rh-123 do not. These results were confirmed histologically. Yet, entry of Lucifer Yellow into the eye is only measurable when the concentration is high enough (Figs 5 and 6). The different behaviour of Procion Yellow and Lucifer Yellow is probably due to the known covalent binding of Procion Yellow to cytoplasmic macromolecules, whereas Lucifer Yellow stabilizes in cells only after aldehyde fixation (Stewart, 1978). Shaw (1977) suggested that the exclusion of Procion Yellow from the locust eye is due to its negative charge, but Lucifer Yellow is also negatively charged. Rh-123 is probably taken up by the retinula cells (in the case of injection into the eye) even in darkness, as indicated by an enhancement of the emission from these cells after 2 h, or by cells in the body, because it is known to accumulate in the mitochondria (Bernal *et al.* 1982).

The supposition that the transport process is active rather than due to restricted diffusion is supported by three different inhibition experiments. Nitrogen, known to abolish receptor potentials and pigment migration in insect eyes (Evequoz *et al.* 1983; Payne, 1981; Weyrauther, 1986) and mitochondrial activity in blowflies (Stavenga & Tinbergen, 1983), totally inhibits the decrease of absorbance difference during its application (Fig. 7). In air the animals recover rapidly (Fig. 7). Ouabain slows down the clearing process. The eye of *Calliphora* is known to possess ouabain-sensitive ATPases (Rivera, 1975). Additionally, the transport of dyes out of the eye is inhibited by lowering the temperature.

The molecular mechanisms causing the active transport are not obvious. An active uptake by retinula cells by endocytosis does not fit with the known exclusion of Trypan Blue from viable cells, and the finding that an uptake of Lucifer Yellow by retinula cells occurs only during prolonged illumination with bright light (Wilcox & Franceschini, 1984*a,b*), which cannot have been the case in the present experiments (Fig. 4, each point results from 10 s illumination with weak test lights). Possibly, the basal glia cells, which form the barrier, themselves enable the transport of substances from and into the retina (Shaw, 1977).

The selectivity of the transport process is not clear. It seems to depend neither on a net charge (otherwise Lucifer Yellow would not be transported) nor on the relative molecular mass: Trypan Blue ( $M_r$  961) is transported, but Rh-123 ( $M_r$  417) and Procion Yellow ( $M_r$  681) are not. In locusts, even inulin ( $M_r$  5200) is

transported from body to eye (Shaw, 1977), and in flies microspheres of 0.2  $\mu\text{m}$  in diameter (fluorescent latex beads) migrate overnight from the retina (injection into a corneal hole) into the abdomen (M. Wilcox & N. Franceschini, personal communication). All these dyes or tracers have high relative molecular masses compared with the common cellular ions. Phenol Red ( $M_r$  354) and sucrose ( $M_r$  342), both substances of low relative molecular mass, are transported very well (see results and Shaw, 1977). By inference, smaller substances such as simple sugars, ions and amino acids will be transported at least as well.

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