Rhodamine B is a lipid-soluble, nontoxic dye that fluoresces at longer wavelengths than fluorescein and consequently is detectable at lower concentrations in the ocular tissues. Its dynamics after topical and systemic administration are similar to those of lipid-soluble drugs. Invest Ophthalmol Vis Sci 25:758–762, 1984

Quantitative measurements of fluorescein in the eye are widely used in studying the function of the blood–retinal barrier, but they also can be of value in measuring the flow rate of the tears and aqueous humor and the barriers offered by the corneal layers and the other anterior structures of the eye. Concentrations down to 10⁻⁹ g/ml can be determined in the ocular humors, and this allows measurements to be made with systemic doses of low toxicity and with topical application. However, the autofluorescence of the lens and the cornea is similar in color to the light emitted by fluorescein, and this raises the threshold of detection of the dye within these tissues. In consequence, a number of potentially valuable investigative techniques cannot be used in humans. The use of a fluorescent compound similar in chemical properties to fluorescein, but with its excitation and emission spectra shifted toward the red, would remove these limitations.

Such a dye is rhodamine B, which is related to fluorescein by the addition of ethylamine groups and the removal of one carboxyl (Fig. 1). We, therefore, studied its behavior in the eye of the rabbit after topical and systemic administration. This was noted to be markedly different from that of fluorescein but similar to that of various drugs whose kinetics have been measured. The physicochemical properties of rhodamine were examined, and its behavior was considered to be a consequence of its high lipid solubility.

Materials and Methods

The investigations utilizing animals, as described in this manuscript, conform to the ARVO Resolution on the Use of Animals in Research.

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Female New Zealand white rabbits weighing 2–3 kg were used in the kinetic studies. The pupil was dilated with atropine and phenylephrine before the fluorometric measurements, and the animals were initially anesthetized with 20 mg/kg of chlorpromazine and 100 mg/kg ketamine; if necessary, the anesthesia was maintained by further doses.

Measurements of fluorescence in test solutions or from the eye were carried out with a simple fluorometer based on the design of Waltman and Kaufman. A 931 photomultiplier was mounted directly onto one eyepiece tube of a Haag-Streit 360 slit lamp. A slit about 6 mm × 0.15 mm was placed at the focal plane of the eyepiece just in front of the photomultiplier entrance aperture. This was equivalent to a collection area from the eye tissue of 2 mm × 0.05 mm. The slit light was provided with a short-wave pass interference filter transmitting 50% of the light at 560 nm, backed with a Kodak 15 gelatin filter, and the barrier filter was a Kodak 24 gelatin filter.

Results

Chemical Properties

The absorption and emission spectra are similar to those of fluorescein but are shifted about 65 nm towards longer wavelengths (Fig. 2). It has a deep red color with an extinction constant of 12.6 × 10⁴ L/mole cm at the wavelength of maximum absorption, similar to the value for fluorescein. The quantum efficiency of the conversion of absorbed to fluorescent light is given as 97%, but this figure applies to the solution in ethanol. A direct comparison of alcoholic and aqueous solutions shows that the latter emits about 55% of the light of the former.

The solubility of rhodamine was measured in water at room temperature at different pH’s. The value 1 g/100 ml is low compared with fluorescein, but unlike fluorescein it does not change with the reaction of the solution, at least over the range pH 5–10. The fluorescence of a dilute solution is fairly constant from pH 4–14, but it falls off in more acid solutions (Fig.
3). The dye is very soluble in 1-octanol and a measurement of the octanol-water partition coefficient gave a value of close to 100:1 at pH 7. Like fluorescein it is bound by plasma protein, and from equilibrium dialysis of rabbit plasma against saline at pH 7 and room temperature, the concentration of bound dye was found to be from six to seven times that of the free dye in the range $10^{-6}$ to $10^{-4}$ g/ml.

Although the formula would suggest that the dye molecule existed as a single- or double-charged cation, it was found scarcely to migrate during iontophoresis on agarose at pH 7; in three experiments a movement of about 0.15 cm$^2$/hr V was noted towards the cathode as opposed to about 2 cm$^2$/hr V for fluorescein in the opposite direction.

**Topical Dynamics**

A drop of 1% rhodamine was instilled into an eye in a number of rabbits and the penetration of the dye observed in the slit lamp. Very shortly after the instillation, the corneal epithelium was seen to be brightly fluorescent, while the conjunctival epithelium was stained a bright pink. In a few minutes, the fluorescence spread into the corneal stroma, where it was seen to develop a gradient from the epithelium to the aqueous humor. After 1 or 2 hr, the epithelium could no longer be distinguished from the stroma, which appeared to be uniformly fluorescent.

The aqueous humor showed a low level of fluorescence, and what was most remarkable was the rapid staining of the lens, which very quickly became brighter than the anterior chamber. The fluorescence was, at first, restricted to a fine line on the surface, as if only the capsule or epithelium was taking up the dye, but it then penetrated into the cortex. Finally it spread round to the posterior pole, so that after a few days
In four rabbits, measurements in the tissues of the eye were attempted with the fluorometer after the instillation of a single drop of 1% rhodamine B brought to pH 7 with hydrochloric acid and made equivalent to 300 mM by the addition of dextrose powder. The general appearance of the concentration curves was similar in each of the eyes, but the results could not be averaged satisfactorily because the times when the rapid changes occurred did not coincide. The results of a representative experiment are shown in Figure 4. The maximum values in the corneal epithelium and in the lens could only be studied only by observing the meter while scanning through the tissue, and because of the lack of sophistication of the instrument they will be underestimates. Early measurements from the stroma were not feasible because of the large concentration gradient within it.

**Systemic Dynamics**

The behavior of the rhodamine in the eye after intravenous injection was observed in the slit lamp in
many animals. The most remarkable feature is the rapid uptake of dye by the cornea and, particularly, the lens both of which shine out in contrast to the poorly fluorescent anterior chamber even as little as 10 min after the injection. No particularly high level of fluorescence was noted in the corneal epithelium at any time.

Measurements were taken again, in four animals, after the injection of the dye into an ear vein, and the results in one case are displayed in Figure 5. Blood samples were taken up into heparinized capillaries from the opposite ear, and readings were taken directly from the undiluted plasma after the samples were centrifuged in their collecting tubes.

Toxicity

Lutty⁶ stated that rhodamine B was extremely toxic, but this was not so in our experience, as observed in a previous note.⁷ Intraperitoneal doses of up to 1 g/kg of neutral solution were survived by rabbits, rats, and mice. Larger doses have proved fatal, the cause of death being sterile peritonitis. Six rats and mice were used in these tests, and there seemed to be no point in killing a large number of animals in order to arrive at a better estimate of an LD₅₀. The skin of the animals is temporarily stained a bright pink at these levels. It was noted that the urine was not intensely stained as it is with fluorescein, which indicates that the kidneys are not the major route of elimination of systemic rhodamine.

Up to 50 μl of 1% neutralized solution were injected into the rabbit cornea without causing inflammation beyond that expected from the trauma of the injection. After a few days, the eye appeared normal except for the residual coloration of the lens. The dye stained the epithelium equally with the stroma shortly after the injection, unlike fluorescein under the same circumstances, which leaves the epithelium unstained.⁵

An injection of 20 μl of the same solution was made into the vitreous body of three rabbits. No sign of retinal damage could be seen with the ophthalmoscope or, in one case, on histologic examination, and the pupillary reflex to light remained indistinguishable from that on the uninjected side. An ERG was taken in one animal and was found to be identical with that on the control side.

Although the dye is not acutely toxic, the 1% solution appears to be irritating to the eye, since rabbits squeezed their orbicularis immediately after topical application. In later experiments, a drop of anesthetic was given before applying this concentration. The expectation of permanently staining a volunteer’s lens ruled out testing whether drops at this concentration were painful to the human eye.

Discussion

The longer wavelengths of the absorption and emission spectra of rhodamine B significantly reduce interference from the native fluorescence of the lens,⁸ in comparison with fluorescein (Fig. 2). With the com-

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Fig. 5. Concentration in blood and ocular media of a rabbit after intravenous injection of 20 mg rhodamine B.
bination of filters used in the photometry, this auto-
fluorescence could scarcely be discerned in the slit lamp
by a dark-adapted observer. Comparison of a $10^{-6}$ g/
ml aqueous rhodamine solution with the fluorescence
of the cornea and lens of the human eye by means of
the fluorometer showed that the background levels in
the tissues were of the order of $2 \times 10^{-9}$ and $2 \times 10^{-8}$
g/ml, respectively. This is two orders of magnitude
lower than for fluorescein and shows that dyes with
the characteristics of rhodamine have the potential of
being measured at much lower concentrations in the
ocular tissues.

However, rhodamine B is not a direct substitute for
fluorescein. After topical administration, it differs in
brightly coloring the corneal epithelium, whereas the
small quantity of fluorescein that crosses to the stroma
leaves the epithelium unstained by its passage. The
function of the epithelium as a reservoir will have to
be taken into account when a full treatment of its
kinetics is attempted. The behavior of rhodamine in
the aqueous humor is clearly more complicated than
that of the biexponential curve followed by fluorescein. 
A further notable difference from fluorescein is the
rapid uptake of the dye by the lens.

The curves corresponding to the measurement of
rhodamine, shown in Fig. 4, are reminiscent of those
of pilocarpine and dexamethasone. 

The behavior of rhodamine after intravenous in-
jection also shows marked differences from that of
fluorescein. The concentration in the plasma falls off
more slowly. Most notable is the rapid rise in the level
in the cornea and lens above that in the anterior cham-
ber. Since this occurs in the center of the tissues, far
from any vasculature, it seems that the tissues are ab-
sorbing the dye from the anterior chamber as rapidly
as it enters from the blood.

Again, the behavior of rhodamine is comparable in
general outline with that of certain drugs, in particular
dexamethasone or tetracycline. The lens was in-
cluded in these drug studies but a high level in its outer
cortex was not noted, probably because the average
value from the entire tissue was determined.

It appears, then, that rhodamine B may mimic the
behavior of those ophthalmic drugs that penetrate the
ocular barriers readily because of their lipid solubility.
This is in accord with the large partition coefficient of
the dye into octanol. The behavior of fluorescein is
probably more comparable with that of the lipid-in-
soluble drugs, eg, the aminoglycosides, which penetrate
very poorly. This is difficult to confirm because the
levels of drug reached in the eye are so low that they
have not yet been quantitated. Since most drugs used
in ophthalmology show some lipid solubility, their ki-
netics may be better understood by a study of the
behavior of rhodamine B and similar compounds.

Key words: rhodamine B, pharmacokinetics, intraocular dy-
namics, lens permeability, corneal permeability

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