# Effects of Cardiac Glycosides
# on Electrical Activity in
# the Isolated Retina of the Frog

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ABSTRACT   Ouabain added to physiological salt solutions bathing the isolated frog retina irreversibly abolishes the electrical response to light (the electroretinogram or ERG). The time course of abolition depends on the concentration of ouabain in the medium and the surface of the retina to which it is applied. When the glycoside is placed on the receptor surface, in 7 min the ERG is completely eliminated by $10^{-4}$ M ouabain and more than 90% inhibited by $3 \times 10^{-5}$ M ouabain. The effect is slower at lower concentrations and when the solution is applied to the vitreous surface of the retina. The evidence suggests that abolition of the ERG by ouabain is due principally to inhibition of the active transport of sodium: (a) Structurally modified glycosides which are considerably less potent inhibitors of alkali cation-activated ATPase activity in preparations of frog retinal outer segments are also poorer inhibitors of electrical activity in isolated retinas. (b) Replacing much of the sodium in the medium bathing the retina by choline, Tris, or sucrose significantly protects the retina from ouabain. It is suggested that in a standard sodium environment essentially constant activity of the sodium pump is required to prevent rapid and irreversible change. The cellular sites most critically dependent on the sodium pump have not been identified.

The retinas of several vertebrate species contain an enzyme system that hydrolyzes ATP in preference to other phosphate esters, requires magnesium for activity, is further markedly stimulated by sodium and potassium together but not separately, and is inhibited by ouabain (Bonting et al., 1964; Frank and Goldsmith, 1965). Although much of this retinal ATPase system appears to occur in the outer segments of the receptor cells, it is present in the inner layers of the retina as well. Similar enzymic activity has been found in the membranes of a variety of cells of many species, and there is now considerable evidence to implicate sodium-potassium–stimulated ATPases in the active transport of these ions across cell membranes (Skou, 1965).

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Like other cells, those in the retina might be expected to have a mechanism for pumping out sodium in order to preserve their osmotic integrity. Moreover, in the slender photoreceptors of the squid, light causes transmembrane ionic currents, which are presumed to be causally related to the generation of impulses in the optic nerve, and that require the presence of sodium ions in the external fluid (Hagins, 1965). The vertebrate retina possesses several kinds of cells, and a similar analysis of its current fields is considerably more difficult. Nevertheless, here too sodium ions are required for a normal electrical response to light (Furukawa and Hanawa, 1955; Hamasaki, 1963). Illumination of the receptors probably subjects the neurons and receptor cells to an influx of sodium and perhaps other ions. In view of the small size of many of the synaptic endings and cell processes, with a consequent high ratio of surface to volume, it would not be surprising if in the vertebrate retina the need for a sodium pump is acute.

The experiments described in this paper provide evidence that the normal functioning of the retina is seriously impaired within several minutes after inhibition of the system for sodium transport. Ouabain and certain other cardiac glycosides rapidly abolish the electroretinogram (ERG) of the frog's isolated retina. Two lines of evidence support the hypothesis that ouabain is primarily affecting the transport of sodium: (a) in comparing the inhibitory effectiveness of different glycosides, there is a parallel between the effect on the alkali cation–activated ATPase activity of isolated rod outer segments and on the electrical activity of the whole retina; and (b) the isolated retina can be significantly protected from ouabain if the solution in which it is bathed contains little sodium. Thus in the absence of sodium leaking into the cell, inhibition of the sodium transport system has less immediate consequences.

## MATERIALS AND METHODS

*Chemicals* Ouabain (Sigma Chemical Co., St. Louis, Mo.) was dissolved directly in aqueous solution. Other glycosides used were scillaren A (K & K Laboratories, Plainview, N.Y.), hexahydroscillaren A and cymarin (kind gifts of Sandoz Inc.), and 17-α-cymarin (generous gift of Professor Christophe Tamm of the Institut für organische Chemie, University of Basel, Switzerland). Because of their limited solubility in water, these latter four glycosides were initially dissolved in 80% (v/v) ethanol and then diluted to the desired concentration with the appropriate physiological saline.

ATP (Sigma) was the disodium salt. Choline chloride (Eastman Organic Chemicals, Rochester, N.Y.) was purchased in the highest available purity and recrystallized before use. Choline chloride was dissolved in 95% ethanol maintained at 60°C until the solution was saturated. Undissolved crystals were removed by filtration, and the supernatant was cooled in a refrigerator overnight without agitation. The crystals were collected by filtration over suction and washed with anhydrous ethyl ether.

*Electrophysiological Experiments* Adult leopard frogs (*Rana pipiens*) of both sexes, obtained throughout the year, were used in all experiments. Frogs were dark-adapted for at least 1 hr before use. They were then decapitated, the eyes removed, and the retinas dissected out under dim room light. Retinas were spread flat on filter paper, usually with the receptor surface facing up, and placed in a Lucite chamber on a fenestrated platform similar to that used by Hamasaki (1963). The chamber was filled to the upper surface of the platform with amphibian Ringer's solution containing 110 mM NaCl, 6 mM $NaHCO_3$, 20 mM glucose, 2.5 mM KCl, and 2.2 mM $CaCl_2$. The compositions of the low-sodium Ringer solutions are given in Table II. The pH was 7.7. In solutions containing ethanol, the final concentration was 4% by volume. A drain at the bottom of the chamber enabled solutions to be removed as desired. Oxygen was constantly bubbled into the solution. Experiments were conducted at a temperature of 25°C. The recording electrode was a fine, L-shaped chlorided silver wire resting lightly on the upper surface of the retina. A silver:silver chloride reference electrode contacted the bath through a cotton wick saturated with Ringer's and led to ground through a voltage calibrator. The amplifiers were usually direct-coupled, but in several experiments were condenser-coupled, with a time constant of 0.6 sec. A solenoid-operated camera shutter admitted the light stimulus, which had a duration of 1 sec. In most cases the stimulus was white light from a tungsten filament microscope lamp. It uniformly illuminated the preparation at about 30 lux. Electrical responses were displayed on the oscilloscope screen and were photographed.

After a retina had been mounted in the chamber a response was recorded and the preparation was left in total darkness for about 10 min. By this time, the ERG had usually reached its maximum control amplitude. The test solution was then applied by one of two methods: (*a*) 5 drops were placed directly onto the exposed surface of the retina at 5 min intervals, or (*b*) the solution was drained from the chamber, test solution was added to cover the retina, drained, the retina was immersed in fresh test solution for 1 min, and finally the solution was drained to the level of the upper surface of the platform and a recording obtained. Responses were recorded every minute or, in longer experiments, every 5 min. When standard sodium Ringer's without additives was the test solution, the electrical response of the retina to light remained unchanged in amplitude and wave form for at least 2 hr. Recordings of electrical responses follow the usual convention, in which a positive potential at the recording (retinal) electrode causes an upward deflection of the trace. For this reason, experiments in which the receptor surface of the retina was oriented upward, in contact with the recording electrode, yielded ERG's which appear upside down, with positive a-waves and negative b-waves. Unless otherwise specified, the receptor surface of the retina faced upward in all experiments.

*Enzyme Assays* Frogs were dark-adapted for at least 1 hr, decapitated, and the retinas isolated. Retinal outer segments in batches from 36 frogs were isolated by homogenization and differential centrifugation as described previously (Frank and Goldsmith, 1965). Isolated outer segments were washed in 50 mM Tris-HCl buffer, pH 7.2, lyophilized, and stored in the freezer. Assays of ATPase activity were performed by an adaptation of the method used previously (Frank and Goldsmith, 1965). The assay medium contained 60 mM sodium, 20 mM potassium, 3 mM magnesium, 1

mM cysteine, 0.1 mM EDTA, and 67 mM Tris buffered to pH 7.2 with HCl. All cations were added as the chlorides. ATP was dissolved in water and buffered to pH 7.2 with Tris. Its final concentration in the assay mixture was 3 mM. Ouabain was added
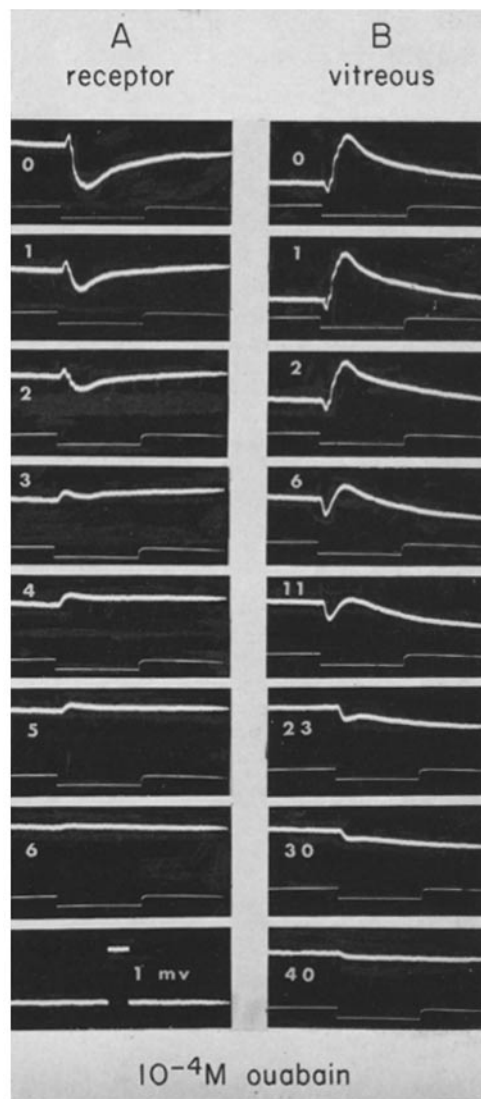


FIGURE 1. Electroretinograms of two isolated frog retinas following application of ouabain at a concentration of $10^{-4}$ M. The numeral at the beginning of each trace gives the time in minutes after placing several drops of glycoside (in Ringer's) on the upper surface of the retina. The upper surface was the receptor side in A, and the vitreous side in B. An upward deflection of the trace indicates that the upper surface became positive to the electrode in the bath. Duration of the stimulating flash, indicated by the lower trace of each pair, was 1 sec. Note that at this concentration of ouabain the b-wave disappears somewhat faster than the a-wave, and abolition of the response is much faster when the glycoside is applied to the receptor rather than to the vitreous surface.

in aqueous solution, but because of their limited solubility, other glycosides were tested in a medium containing 0.4 % (v/v) ethanol. This permitted a maximal glycoside concentration of $10^{-5}$ M, in contrast to the $10^{-4}$ M used in the electrophysiological experiments, where solutions containing 4 % ethanol were employed. In preparations of lyophilized outer segments, however, 4 % ethanol significantly inhibited ATPase activity. The powder of outer segments was suspended in distilled water and homogenized to give an even suspension containing 10 mg dry weight of tissue per ml. One·

tenth ml of this suspension was placed in the reaction tubes, ATP added, and the tubes incubated for 30 min at 37°C at a final volume of 0.75 ml. The reaction was terminated by adding 1.5 ml of ice-cold 10% (w/v) aqueous trichloroacetic acid. In all experiments, tubes containing ATP but no enzyme were incubated to estimate non-enzymatic hydrolysis, and tubes containing ATP and enzyme but no glycoside and no potassium were incubated to estimate the theoretical maximal glycoside inhibition. This was necessary because the highest glycoside concentration obtainable with 0.4%
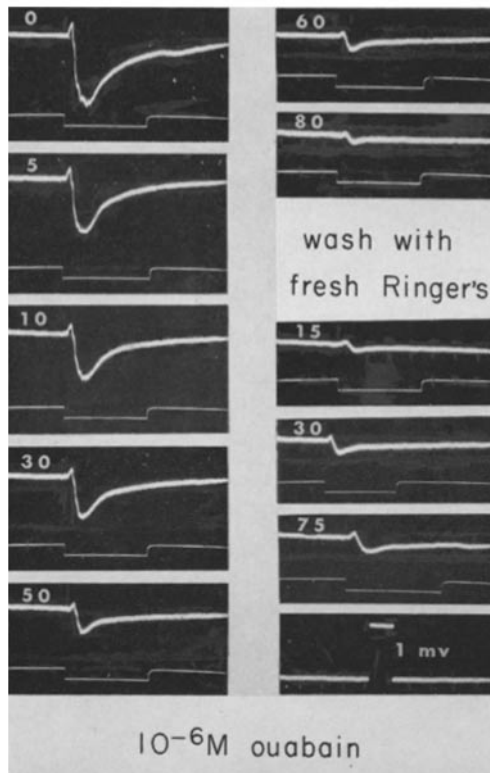


Figure 2. Time course of effect of $10^{-6}$ M ouabain applied to the receptor surface of an isolated frog retina. Details of recording as in Fig. 1. At this concentration, the a- and b-waves decline in parallel. Note that after 80 min of exposure to ouabain the retina was nearly unresponsive, and washing with fresh Ringer's produced very little recovery after more than an hour.

ethanol does not inhibit maximally. Since maximal inhibition by glycosides eliminates all the sodium-potassium-stimulated activity, one can determine maximal inhibition simply by omitting potassium from the medium.

After adding trichloroacetic acid, the tubes were centrifuged and 1 ml of each supernatant was mixed with 1 ml of a solution of 1% (w/v) ammonium molybdate and 4% (w/v) $FeSO_4$ in 1.15 N $H_2SO_4$. Optical density was measured at 700 nm, and the phosphate concentration determined from a standard curve. The preparations of outer segments were doubtless contaminated with mitochondria, but the ouabain-sensitive component of ATPase activity is not of mitochondrial origin (Frank and Goldsmith, 1965). The preparations are referred to as isolated outer segments with the realization that small membrane fragments from other retinal structures could contribute activity.

RESULTS

I. *Effects of Ouabain on Electrical Activity*

Ouabain abolishes electrical activity in the isolated retina. When applied to the receptor surface at a concentration of $10^{-4}$ M in a standard Ringer, the b-wave was gone after 4 min, and within 7 min the retina was unresponsive (Fig. 1 A). If the preparation was now washed repeatedly by immersion in fresh Ringer's without glycoside, electrical activity could not be restored.

A similar sequence of events was observed when $10^{-4}$ M ouabain in Ringer's was applied to the vitreous surface of the retina, but the time course was significantly slower, requiring more than 40 min. to abolish the ERG (Fig. 1 B). Once again, the effect of ouabain was irreversible.

At an even higher concentration ($10^{-3}$ M) abolition of activity was still faster than shown in Fig. 1, and the isolation of the persistent negativity known as PIII (Granit, 1933) was more complete. When ouabain was applied to the receptor surface, the b-wave began to diminish within less than a minute, and by 2 min only PIII remained.

Lower concentrations of ouabain have similar effects but act more slowly. The lowest concentration of ouabain that consistently abolished the electrical response of the retina was $10^{-6}$ M. At this concentration, more than 100 min was required to eliminate the ERG. Both the a- and b-waves diminished together, so the isolated PIII did not appear. In the experiment shown in Fig. 2, the ERG had declined to about 10 % of its initial amplitude 80 min after $10^{-6}$ M ouabain had been applied to the receptor surface. At this time, the retina was immersed in fresh Ringer's without glycoside. A response was recorded every 5 min thereafter, and was immediately followed by washing the retina in fresh Ringer's. This experiment shows that even when the ERG has not been completely abolished, repeated washing produces very little recovery.

When ouabain in concentrations of $10^{-7}$ M and lower was applied to the retina, electrical activity was not inhibited. The ERG retained its original form and amplitude for at least 2 hr after the initial application of the test substance, which was reapplied every 5 min for the duration of the experiment.

II. *The Nature of the Inhibitory Effect of Cardiac Glycosides*

A possible explanation for the abolition of the ERG is that ouabain is inhibiting sodium transport. If this is true, one would expect the transmembrane gradient of sodium to run down, and eventually cells to be damaged by inward leakage of sodium chloride and osmotic uptake of water. Two kinds of experiments were performed which support the interpretation that ouabain affects the sodium pump.

EFFECTS OF OTHER GLYCOSIDES ON ELECTRICAL ACTIVITY    The experiments of Glynn and his associates (Glynn, 1957; Dunham and Glynn, 1961) have demonstrated structural requirements in the glycoside molecule for inhibition of both active sodium transport and sodium-potassium–stimulated ATPase activity in the membranes of human erythrocytes. The glycosides scillaren A and cymarin are potent inhibitors of both transport and ATPase activity. When the lactone ring at carbon-17 is hydrogenated, as in hexahydroscillaren A, or when it has the $\alpha$-orientation relative to the steroid nucleus, as in 17-$\alpha$-cymarin (Fig. 3), inhibitory effectiveness in both these assays
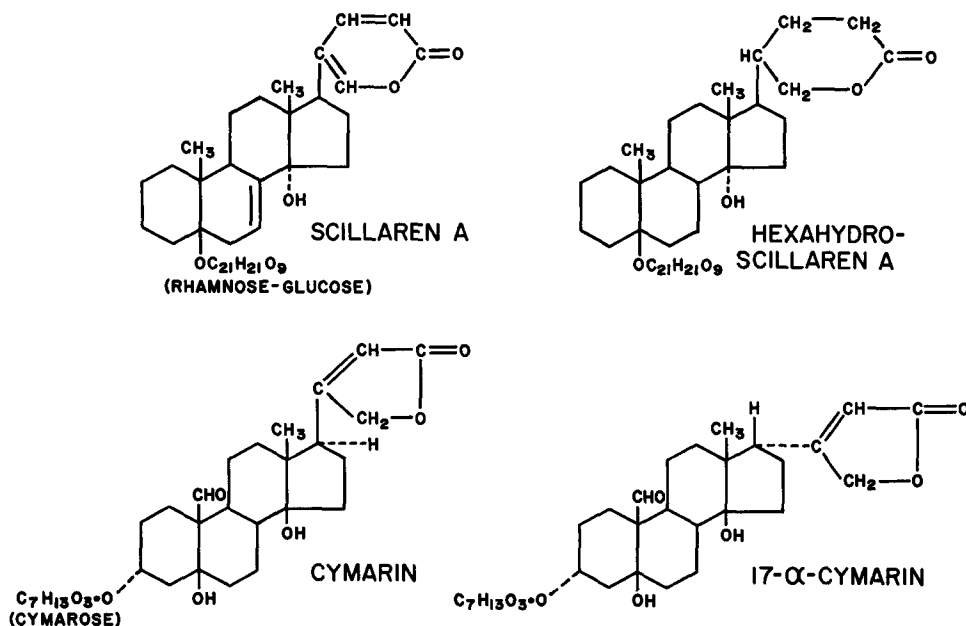


FIGURE 3.    Structural formulas of four cardiac glycosides whose inhibitory effects were tested both on the electrical response of the isolated retina and on the ATPase activity of isolated outer segments of the receptors.

is considerably reduced. Similar structural requirements for inhibition of electrical activity in the isolated frog retina would suggest a relationship between the sodium-potassium–activated ATPase system in the retina and excitability.

Experiments were carried out in which retinas were exposed to these glycosides at a concentration of $10^{-4}$ M in amphibian Ringer's. Because 4 % ethanol is known to alter the amplitude and wave form of the ERG (Bernhard and Skogland, 1941), control retinas were treated with 4 % ethanol in Ringer's without glycoside. Fig. 4 shows records from a sample experiment involving two of these glycosides. The column on the left is a series of responses of a control retina incubated in 4 % ethanol in Ringer's. Shortly after application of the
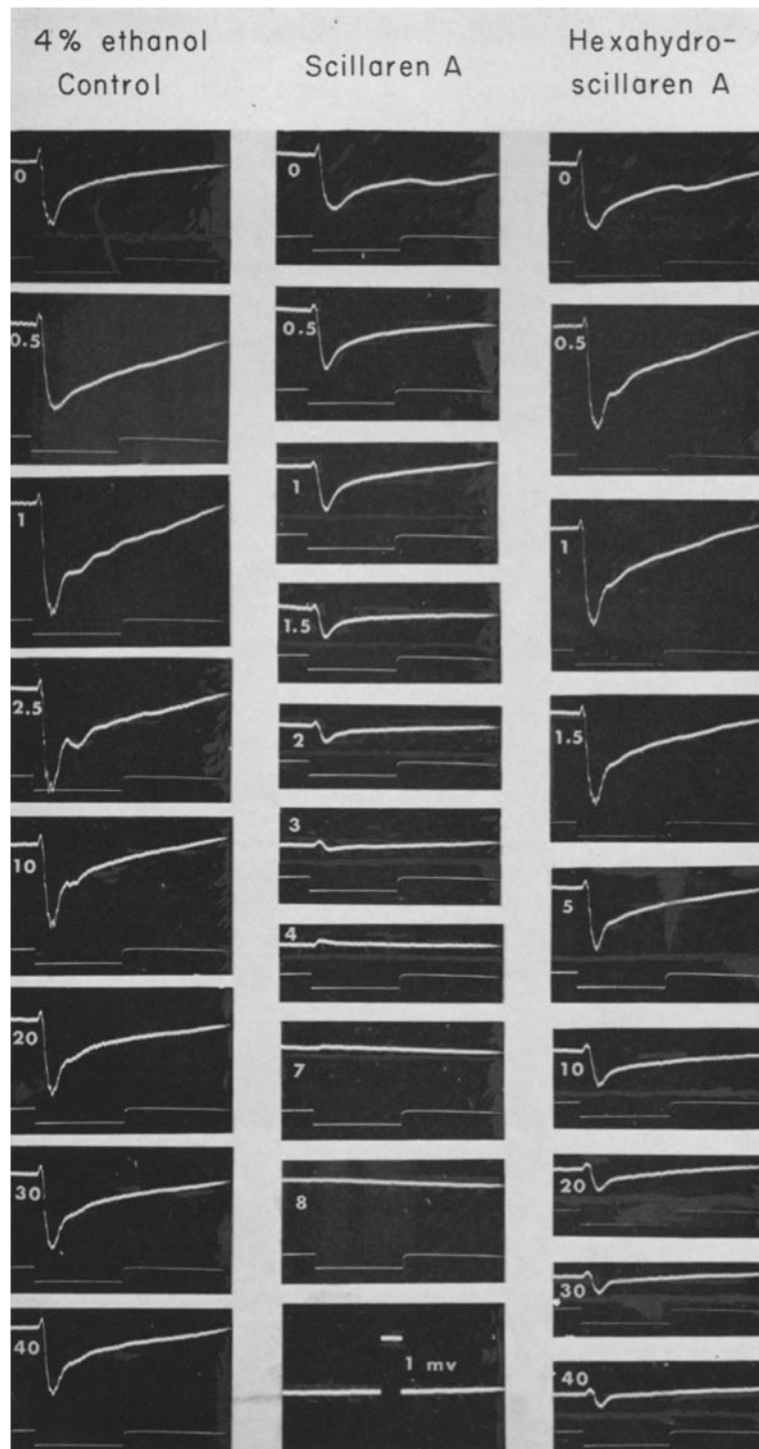
FIGURE 4. Effects of 4 volumes % ethanol in Ringer's, $10^{-4}$ M scillaren A in 4% ethanolic Ringer's, and $10^{-4}$ M hexahydroscillaren A in 4% ethanolic Ringer's. Test solutions were applied to the receptor surface. Each vertical column of oscillograms was recorded from a separate retina. Other details of recording are as described in the legend to Fig. 1.

test solution, the b-wave increased in amplitude and the a-wave diminished slightly. The wave form of the b-wave also changed noticeably. Over a period of time, despite addition of fresh ethanol-Ringer's to the retina at 5 min intervals, the amplitude of the b-wave declined, and in most experiments it was somewhat smaller than its original size after 40 min. When $10^{-4}$ M scillaren A in 4 % ethanolic Ringer's was added to the retina, however, the effects were much different (Fig. 4, middle column). No initial increase in the amplitude of the b-wave was seen; instead the electrical response began to decline rapidly; and by 8 min the ERG had disappeared irreversibly. When applied
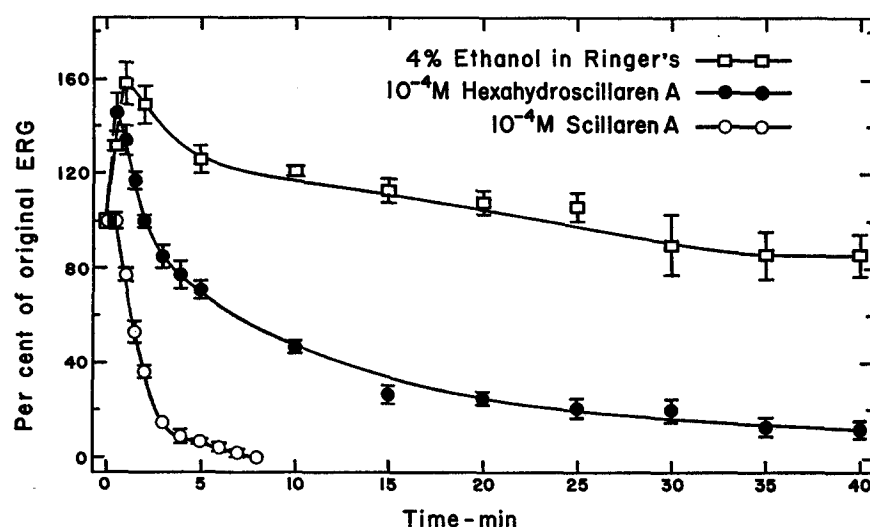


Figure 5. Mean rates of effect of hexahydroscillaren A and scillaren A, based on a series of experiments similar to that shown in Fig. 4. Each point is an average of four retinas.

at a concentration of $10^{-4}$ M, hexahydroscillaren A also abolished the ERG, but its action was much slower (Fig. 4, right-hand column). The initial, ethanol-induced enhancement of the b-wave was seen, followed by a gradual decline in the response, which required 55 min to disappear entirely.

Fig. 5 shows graphically the mean results of four experiments in each of these solutions. Each response is plotted as a percentage of the control trace, recorded at zero time. Both a- and b-waves are summed together for this measurement.

When $10^{-4}$ M cymarin and 17-$\alpha$-cymarin were applied to isolated frog retinas, the results were similar (Fig. 6). Cymarin abolished the electrical response to light very effectively, requiring 7 min to render the retina completely unresponsive. 17-$\alpha$-cymarin was considerably less effective, requiring 25 min to produce the same result.

Fig. 7 shows the method used to compare these four glycosides quantitatively. The filled circles and the curve represent the rate (reciprocal time) at which ouabain abolishes the ERG, plotted as a function of ouabain concentration. Each point is an average of five or more experiments. The average rates for the other four glycosides are plotted as crosses on the "dose-response" curve for ouabain. By dropping perpendiculars from these points to the ab-
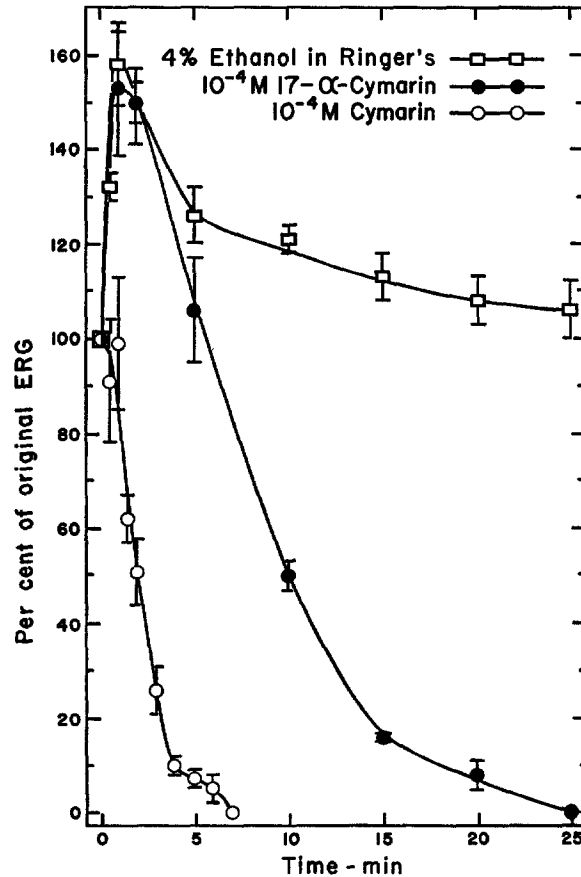


FIGURE 6. Average kinetics of inhibition of the electroretinogram by cymarin and 17-α-cymarin. Each point is based on an average of four retinas. Glycosides were applied to the receptor surface in 4% ethanolic Ringer's.

scissa, one obtains the concentration of ouabain that produces the same effect as $10^{-4}$ M of the test glycoside. The end point, total abolition of the response, is arbitrary, so similar curves were constructed, using reduction of the response to 75, 50, and 25 % of the control as the endpoint. Each gave a similar result. Concentrations of ouabain equivalent to $10^{-4}$ M of the other four glycosides were averaged from these four curves (the latter three are not shown), and the mean values ± SE are given in Table I. The similarity of values obtained

from these four curves demonstrates that no prominent features were obscured by lumping the a- and b-waves together.

INHIBITION OF ATPASE ACTIVITY BY GLYCOSIDES    When one plots enzymatic activity as a function of the logarithm of the ouabain concentration
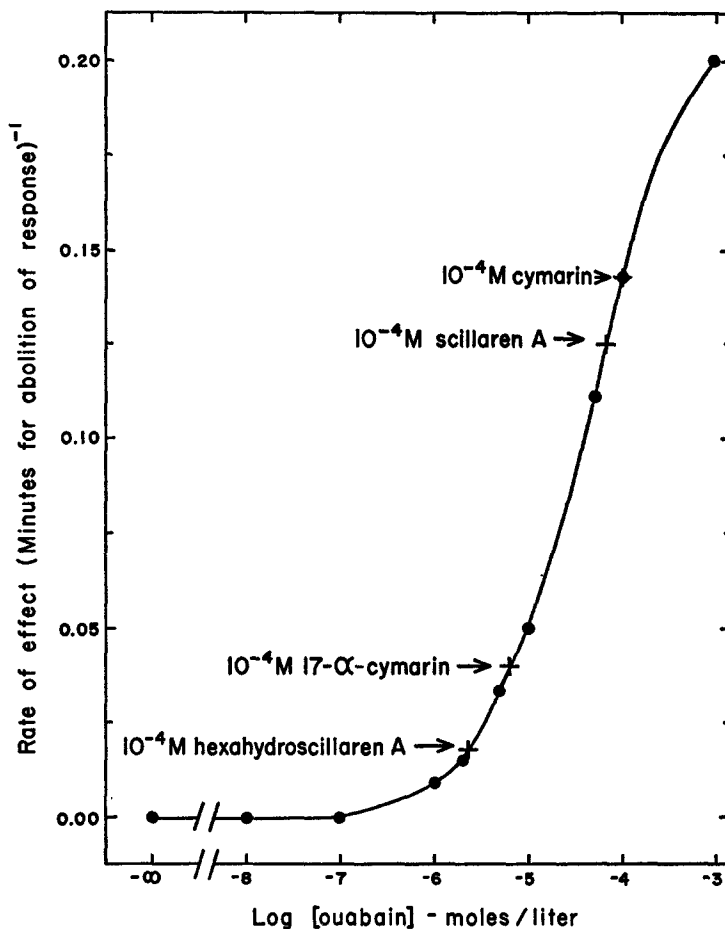


FIGURE 7.   Method used for the quantitative comparison of the inhibitory effects of four cardiac glycosides on the electrical activity of the isolated retina. Filled circles and solid curve show reciprocal time for abolition of the ERG in various concentrations of ouabain. Rate of abolition of the ERG by $10^{-4}$ M of each of four other glycosides is shown by crosses on the dose-response curve for ouabain. The concentration of ouabain equivalent to each of the test substances can be read from the abscissa and is given in Table I. See the text for further discussion.

(Fig. 8), a typical sigmoid curve is obtained. For frog retinal outer segments, the concentration for half-maximal inhibition (arrow) is $1 \times 10^{-7}$ M, a figure of the same order of magnitude as the $6.3 \times 10^{-7}$ M previously determined for pig outer segments (Frank and Goldsmith, 1965). The total ATPase activity

was about 2.50 mmoles phosphate released per gram dry weight of tissue per 30 min. This is about half the total activity found in preparations of pig outer segments under the same assay conditions (Frank and Goldsmith, 1965), but is very similar, when corrected to the same units, to the figures for frog ob-

TABLE I

RELATIVE EFFECTIVENESS OF FOUR GLYCOSIDES IN ABOLISHING ERG OF ISOLATED FROG RETINAS AND AS INHIBITORS OF $Na^+$-$K^+$ ATPase OF FROG RETINAL OUTER SEGMENTS

| Glycosides | Concentration of ouabain (M) required to produce same effect as $10^{-4}$ M glycoside | Relative inhibitory effectiveness on electrical response* | Concentration (M) required to produce half-maximal inhibition of ATPase | Relative inhibitory effectiveness on ATPase* |
|---|---|---|---|---|
| Scillaren A | $(9.2 \pm 0.8) \times 10^{-5}$ | 100 | $2.0 \times 10^{-8}$ | 100 |
| Cymarin | $(6.8 \pm 1.4) \times 10^{-5}$ | 74 | $2.0 \times 10^{-8}$ | 100 |
| Hexahydroscillaren A | $(7.1 \pm 2.0) \times 10^{-6}$ | 7.7 | $5.0 \times 10^{-6}$ | 0.4 |
| 17-α Cymarin | $(6.9 \pm 0.0) \times 10^{-6}$ | 7.5 | $2.5 \times 10^{-6}$ | 0.8 |

* Based on scillaren A as 100.
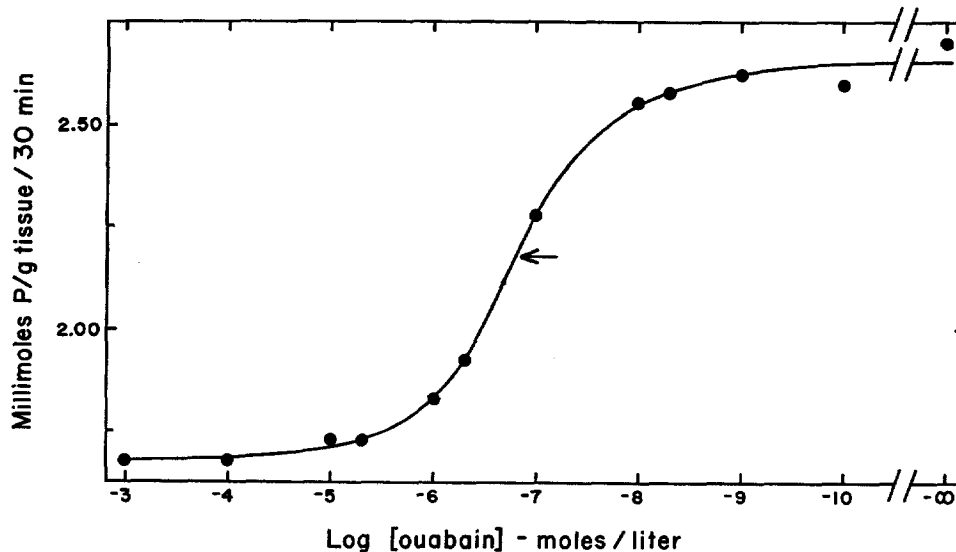


FIGURE 8. Inhibition by ouabain of ATPase activity in the preparations containing outer segments of frog retinal receptors.

tained by Bonting et al., (1964) using a slightly different assay. On the other hand, we find that only about 33 % of the total ATPase activity is stimulated by sodium and potassium and inhibited by glycosides, in contrast to approximately 50 % of the total in pig under the same conditions (Frank and Goldsmith, 1965), and 67 % in frog reported by Bonting et al. (1964).

Inhibition by scillaren A, hexahydroscillaren A, cymarin, and 17-α-cymarin is shown in Figs. 9 and 10. Points are averages ±se for four determinations. Total inhibition (see Methods) is shown by the X's. The data were more scattered than with ouabain, perhaps because of the presence of ethanol. The curves were drawn by eye. The concentrations of glycosides for half-maximal inhibition (arrows) are given in Table I.
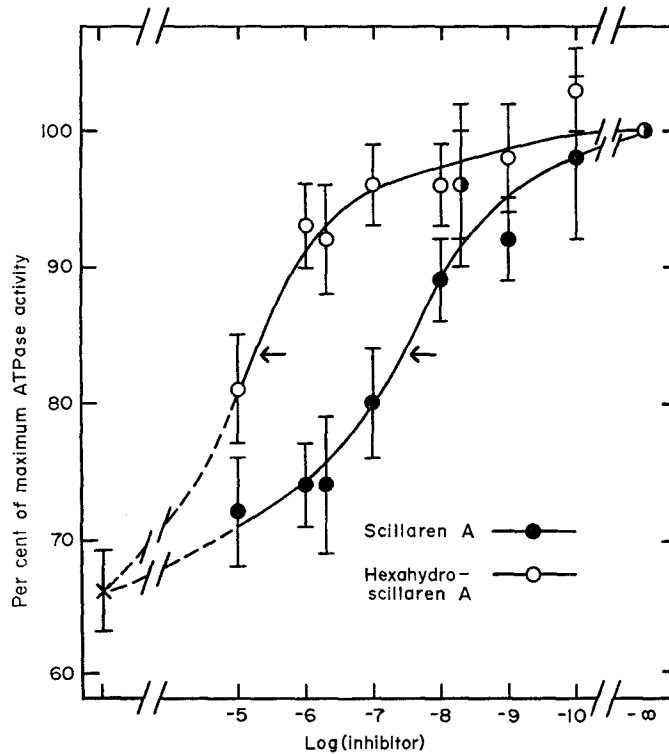


Figure 9. Inhibition by scillaren A and hexahydroscillaren A of ATPase activity in preparations of receptor outer segments. Each point is an average of four experiments ± the standard error. Total inhibition (cross) was estimated from experiments in which potassium was omitted from the incubation medium. Half-maximal inhibition is shown by the arrows.

THE EFFECT OF OUABAIN IN LOW SODIUM SOLUTIONS  If the inhibitory effect of cardiac glycosides on the electrical activity of the isolated retina is caused by interference with sodium transport, it should be possible to protect the retina from the action of ouabain by simultaneously preventing the inward leakage of sodium. This can be done by lowering the concentration of sodium in the external solution, replacing it with a relatively impermeable species. Several experiments of this nature were performed, in which most of the sodium was replaced by choline, Tris, or sucrose.

Retinas were incubated in the dark in normal sodium Ringer's without glycoside until electrical responses were stable, and the solution was then drained from the chamber. The retina was quickly washed once with test solution, immersed in fresh test solution for 1 min, and then left moist with test solution for a total contact time of 6 min. Several responses were obtained during the last few minutes. The test solution was then drained from the chamber and the retina was immersed for 1 min in fresh, standard Ringer's with-
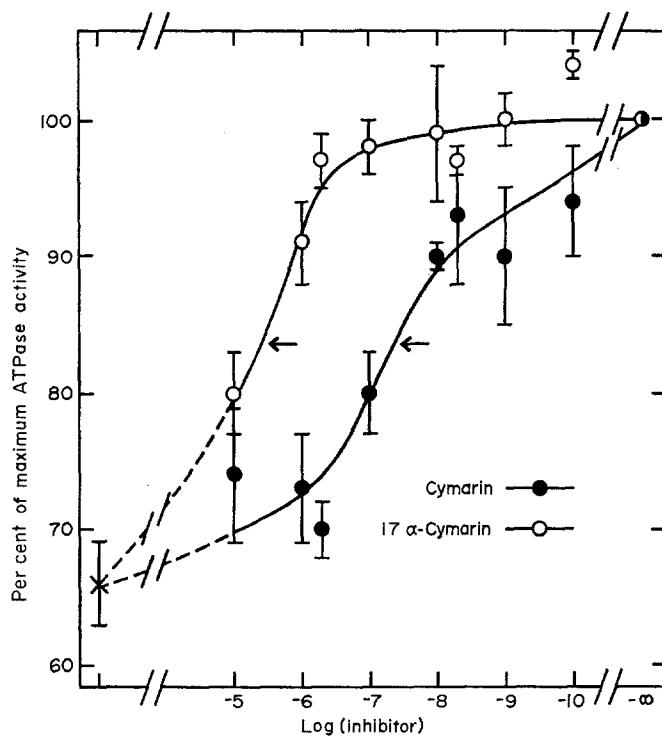


FIGURE 10.    Inhibition by cymarin and 17-α-cymarin of ATPase activity in preparations of receptor outer segments. Each point is an average of four experiments ± the standard error. Total inhibition (cross) was estimated from experiments in which potassium was omitted from the incubation medium. Half-maximal inhibition is shown by the arrows.

out glycoside. The retina was kept in contact with standard sodium Ringer's for an additional 15–20 min, and responses were recorded. Periodically during this time of recovery, more fresh Ringer's was dripped on the retina to wash it.

Fig. 11 shows the results of a typical experiment done in choline Ringer's, as well as two controls. When normal sodium Ringer's containing $3 \times 10^{-5}$ M ouabain was added (squares), the amplitude of the electrical responses declined over the incubation period, until, at the end of 6 min, the ERG was less than 10 % of its original size. Reincubation in fresh Ringer's without glyco-
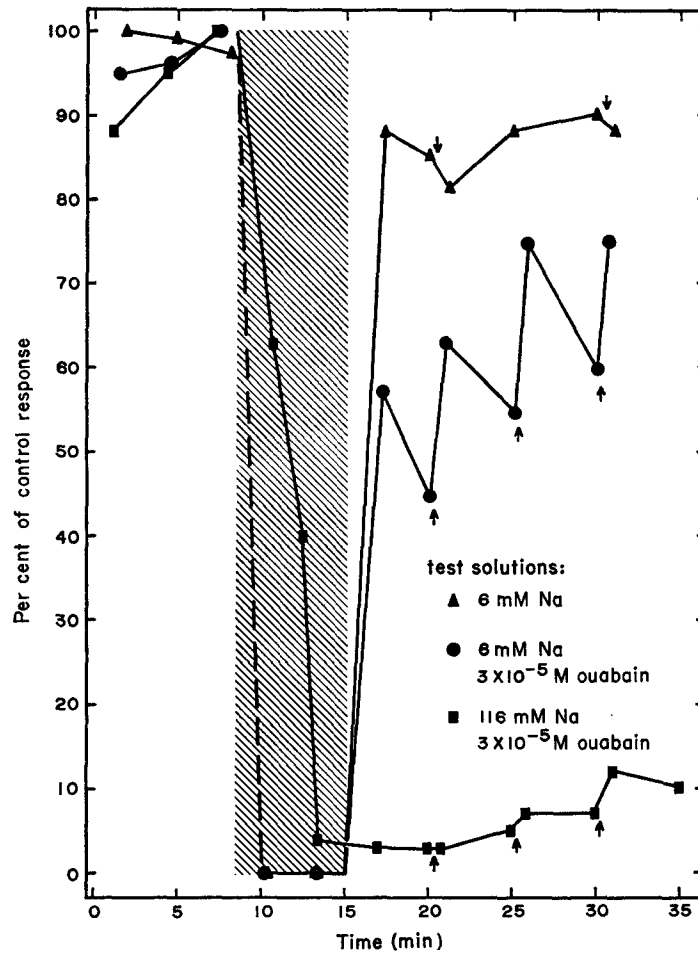
Figure 11. Protection of the isolated retina from the effects of ouabain by low sodium, choline Ringer's. Each of the three curves is a single experiment done on one retina. During the time indicated by the shaded area of the graph, the retinas were exposed to the test solutions, and during the first minute of this period they were totally immersed. During the times indicated by the unshaded regions of the graph, the lower (vitreous) surface of the retina was in contact with standard sodium Ringer's. At the times indicated by the small arrows, the retinas were washed by dripping standard sodium Ringer's onto the upper (receptor) surface. See the text for a fuller description of this experiment.

side increased the response but little, despite repeated washings (arrows). When low sodium Ringer's without glycoside was added, the response was promptly abolished (triangles).[1] When the retina was returned to fresh Ringer's after 6 min, the ERG returned with its original wave form and with

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[1] Reversible abolition of the vertebrate ERG in choline chloride has been reported previously (Hamasaki, 1963) and is to be expected if the response represents receptor and postsynaptic potentials with conventional ionic bases.

nearly its original amplitude. Repeated washings in fresh Ringer's did not significantly alter the form or amplitude of the response. With low sodium Ringer's containing $3 \times 10^{-5}$ M ouabain, the ERG also disappeared rapidly upon addition of the test medium, and after return to fresh sodium Ringer's, the response came back with its original wave form and 70–80 % of its original amplitude (circles). Unlike the situation when low sodium Ringer's alone was added, however, the response began to decline with time. The amplitude could be restored, though, by washing with fresh Ringer's (arrows). This "sawtooth" recovery curve was consistently seen in experiments with choline Ringer's and ouabain.

TABLE II

PROTECTION FROM OUABAIN BY LOW SODIUM

| Test Ringer's solution | Contents | Na | Ouabain | Recovery (per cent of the pretreated control ± SE) |
|---|---|---|---|---|
| | $mM$ | $mM$ | $M$ | |
| "Sodium" | Cl⁻, 116.9 | 116 | $3\times10^{-5}$ | $8.2\pm1.7$ |
| "Choline" | Choline, 110 | 6 | 0 | $85.4\pm8.2$ |
| | Cl⁻, 116.9 | 6 | $3\times10^{-5}$ | $73.2\pm4.9$ |
| "Tris" | Tris, 110 Cl⁻, 94.4 | 6 | 0 | $128.5\pm13.3$ |
| | Glucose, 25 | 6 | $3\times10^{-5}$ | $55.8\pm2.9$ |
| "Sucrose" | Sucrose, 220 | 6 | 0 | $70.5\pm6.7$ |
| | Cl⁻, 6.9 | 6 | $3\times10^{-5}$ | $29.6\pm2.6$ |

In addition to the substances listed in the first two columns, each Ringer's contained $HCO_3^-$, 6 mM; $Ca^{++}$, 2.2 mM; $K^+$, 2.5 mM; and glucose, 20 mM.

Table II summarizes the results of a number of such experiments. Each entry under "recovery" is the average of five determinations on separate retinas. Recovery is based on the largest response obtained during the 15–20 min of washing which followed exposure to the test solution, and is expressed as a per cent of the amplitude of the untreated control measured prior to immersion in the test solution. Ouabain clearly has little if any effect when presented in the low sodium, choline Ringer's.

Choline Ringer's protects the retina against ouabain only so long as the low sodium solution is present. When a retina was incubated in choline Ringer's and then permitted to recover its excitability in standard sodium Ringer's, subsequent addition of sodium Ringer's containing ouabain abolished the electrical response with the same time course as in retinas that had never been exposed to choline. This experiment excludes the possibility that choline may prevent the effect of ouabain by causing some long term change in the retina.

To see whether this protection from ouabain is a result of decreased sodium

rather than the presence of choline per se, similar experiments were done in Tris and sucrose Ringer's (Table II). Tris is not inert. In its presence the response initially vanished, but a small ERG could be recorded before the end of the 6 min test period. On washing, the response was potentiated, superficially resembling the effects of ethanol. This is the reason why responses after Tris (without ouabain) were nearly 30 % larger than the initial controls. Despite these complications, Table II shows that all three low sodium solutions afford a significant measure of protection from ouabain. The effect is not as clear, however, with sucrose and Tris solutions as it is with choline.

## DISCUSSION

*The Mode of Action of Cardiac Glycosides*   There is much evidence that the physiological effects of cardiac glycosides are quite specific (Glynn, 1964). For example, Schatzmann (1953) showed that strophanthin as well as its aglycone inhibited sodium and potassium fluxes in red blood cells, but had no effect on either glycolysis or respiration. Moreover, Caldwell and Keynes (1959) showed that ouabain inhibits the active extrusion of sodium from the giant axon of the squid only when it is applied to the external surface of the membrane. Ouabain injected intracellularly has no effect. Dunham and Glynn (1961) showed that the inhibitory effect of low concentrations of ouabain on the ATPase system of human erythrocyte membranes is reversed by elevated concentrations of potassium. In the same system, external potassium stimulates transport (Glynn, 1957). The similar structural requirements of glycosides for inhibition of ATPase activity and sodium-potassium transport in red blood cells have already been mentioned. Although other actions of cardiac glycosides have also been observed, e.g. inhibition of transport of sugars and amino acids, effects on excitation-contraction coupling in cardiac muscle (for references and discussion see Glynn, 1964), two results of the present experiments strongly suggest that in the isolated retina cardiac glycosides abolish the electrical response to light principally because of their specific inhibition of sodium transport.

As shown in Table I, there is a parallel action of glycosides on both the ATPase activity of outer segment preparations and the abolition of the ERG; those molecules which are effective inhibitions of one serve also to inhibit the other. The most straightforward interpretation is that the primary action of ouabain and other cardiac glycosides on the intact retina is to inhibit the transport of sodium, and disappearance of electrical activity is a consequence of this.

This conclusion, although appealing, is presented gingerly. In the first place, the comparisons in Table I lack quantitative precision. Although the relatively inactive glycosides have only about 1 % the inhibitory effectiveness of their active counterparts in the enzymatic assay, they have roughly 10 % the potency of the inactive forms in the electroretinographic experiments.

Another possible difficulty with the interpretation of Table I is that the ATPase is nearly 50 % inhibited in the presence of $10^{-7}$ M ouabain (Fig. 8), whereas such a low concentration has no significant effect on the electrical response of the whole retina. Put in another way, over the range $10^{-3}$ to $10^{-5}$ M ouabain, the inhibition of enzyme activity is essentially complete (Fig. 8), but there are conspicuous differences in the rates at which $10^{-3}$ M and $10^{-5}$ M ouabain abolish the ERG (Fig. 7). There are at least three possible explanations for this quantitative discrepancy that are compatible with the conclusion that in the physiological experiments the effects of glycosides were due specifically to inhibition of an active cation pump: (a) The transport ATPase system in membrane fragments of lyophilized outer segments suspended in a uniform ionic environment is more sensitive to cardiac glycosides than is the enzyme system of intact, living retinas in which the cell membranes are exposed to different ionic compositions on their two sides. (b) The critical site of action of cardiac glycosides in abolishing the ERG is not in the outer segments but elsewhere in the retina, and is less sensitive to glycosides than the binding site studied enzymatically in the outer segments. Glynn (1964) and Skou (1965), for example, have pointed out that sodium-potassium—activated ATPases from different species and even in different tissues from the same species may vary in their sensitivity to ouabain. (c) Alternatively, in a relatively dark-adapted state (as in these experiments), the retinal cells may have a far greater pumping capacity than necessary to compensate for leakage of sodium down its electrochemical gradient. Consequently, all but a fraction of the pumping capacity might be inhibited in the dark, with but negligible effect on the internal ionic composition of the cell. Inhibition of the remaining transport machinery might then lead rapidly to accumulation of sodium and resultant inexcitability in critical cell structures. Although experiments have not been done to test these possibilities, the third seems particularly interesting. If light excites retinal cells by increasing their permeability to ions, then the presence of an ion pump with a capacity considerably in excess of the demands made on it in the dark may be of great value in restoring and maintaining the sensitivity of the retina during continued exposure to bright light.

A final vexatious point is that 4 % ethanol considerably inhibits the ATPase activity of outer segments but temporarily enhances the electrical responses of the isolated retina. If functioning of the transport system is essential for excitability, any inhibitor of the ATPase system might be expected to abolish the electrical response of the retina. It may be, however, that in vivo the sensitivity of the transport ATPase system to ethanol is considerably less than in a suspension of membrane fragments. In such a lyophilized, homogenized suspension, components of the transport system might be available for attack by ethanol that are not susceptible in vivo when cell structure is intact. Moreover, if indeed there is a slight inhibitory effect of ethanol on the transport

enzymes, this would be difficult to detect in the presence of the enhanced b-wave produced also by ethanol (Fig. 4; Bernhard and Skoglund, 1941). The increased b-wave is thought to result from interference with inhibitory synaptic processes and is probably unrelated to any action of ethanol on sodium transport.

The abolition of responses is not likely to be the effect of a synergistic interaction of ethanol and the glycosides, because the rate of action of $10^{-4}$ M cymarin is about the same as that of $10^{-4}$ M ouabain, and ethanol was present only with the former.

Despite these reservations about Table I, Table II encourages the belief that in the isolated retina cardiac glycosides primarily affect sodium transport. In several kinds of low sodium solution, the electrical responsiveness of the retina is at least partially protected from the deleterious effect of ouabain. The common feature of these test solutions is that all lower the electrochemical gradient of sodium across the cell membranes. The experimental evidence does not rule out additional effects of glycosides, however. For example, relatively nonspecific surface-active properties might cause the degree of permanent damage observed in Tris and sucrose Ringer's. Further evidence in the form of direct measurements of ionic fluxes and observations of fine structure would be helpful.

Abolition of the ERG is irreversible, even after the ouabain has been washed away by repeated applications of fresh Ringer's. If the response has been only partially abolished, it does not diminish further after removal of the ouabain, and may increase somewhat with washing (Figs. 2 and 11). If ouabain becomes irreversibly bound to its site on the membrane, one would expect the response to continue to decrease despite repeated washings. It may be that much of the ouabain can be removed by washing, and that failure to recover fully reflects irreversible damage to a large fraction of the cells, perhaps caused by leakage of sodium chloride and water during inhibition of transport. Swelling and other cytoplasmic changes are shown in Birks' (1962) electron micrographs of nerve cells exposed to digoxin.

*The Rate of Action of Cardiac Glycosides*   The rate at which ouabain and other cardiac glycosides affect the electrical response of the isolated retina is probably influenced both by diffusion of the glycoside to its site of action and the time necessary for the physiological effect to be expressed. That ouabain acts so much more slowly when applied to the vitreous rather than to the receptor surface suggests either an unidentified barrier to diffusion from the vitreous side or a site of action closer to the receptor than to the vitreous surface (see below). Even when ouabain is applied to the receptor surface, however, diffusion may affect the rate. For unimpeded diffusion into the retina from an unstirred drop of test solution, about 20 sec would be required for the glycoside concentration to reach one-third the initial value of the test

solution at a depth of 50 $\mu$m, and more than 1 min at a depth of 100 $\mu$m. Because of uncertainties about the exact path of diffusion, boundary conditions, diffusion coefficient, and the site of action, this calculation must be considered rough; however, it indicates a process whose rate is not much faster than the results observed at $10^{-4}$ M and higher concentrations. On the other hand, the differences in the rates of action of the pairs of structurally modified glycosides illustrated in Figs. 5 and 6 cannot be ascribed to diffusion and must reflect their potencies as inhibitors.

On the premise that the effects of cardiac glycosides on the ERG result primarily from inhibition of the sodium pump, it is significant that in 6 min the electrical response of the retina can be abolished with $10^{-4}$ M ouabain, and 90 % eliminated with $3 \times 10^{-5}$ M. Ouabain ($10^{-5}$ M) applied to the giant axon of the squid promptly slows the active extrusion of sodium, but there is no detectable change in the resting or action potentials (Caldwell and Keynes, 1959). Apparently the surface:volume ratio is so low in these giant fibers that, even without the sodium pump, leakage of sodium causes insignificant changes in the internal concentration over a period of several hours. Even with much smaller cells, Birks (1963) found that digoxin in various concentrations greater than $1.3 \times 10^{-6}$ M required at least half an hour to produce transmission block in the fine motor nerve terminals of frog skeletal muscle.

*The Site of Action of Cardiac Glycosides*    Identification of the cellular site of action of ouabain is not possible from the available evidence and remains the most provocative issue raised by these experiments. Not only ouabain, but also a large variety of substances affect the ERG faster when applied to the receptor rather than to the vitreous surface of the isolated retina (Hamasaki, 1964). Either there is a permeability barrier on the vitreous side, or the sites of action of potassium chloride, sodium azide, formaldehyde, trypsin, and ouabain all lie close to the receptor surface.

Lasansky and Wald (1962) and Lasansky (1965) have studied the fine structure of the toad's retina and looked for barriers to diffusion. The inner limiting membrane is a basement membrane which appears to offer no significant impediment to the movement of small molecules from the vitreous humor into the extensive system of extracellular channels formed by the foldings of the plasma membranes of the Müller cells. The outer limiting membrane (or junctional layer), which is found at the other end of the Müller cells, at the level of the inner segments of the photoreceptors, is, on the other hand, characterized by numerous tight junctions between portions of Müller cells, and small areas of adhesion between portions of Müller cells, Müller cells and receptors, and between receptors. On anatomical grounds, therefore, solutes penetrating the retina from the receptor side might meet a partial barrier in this region of tight junctions and somewhat narrowed intercellular clefts of the adhering zonules. These conclusions were borne out by using the elec-

tron microscope to study the penetration of ferrocyanide into the extracellular space of the retina. Following total immersion of the retina or application of ferrocyanide to the vitreous side, maximal staining was observed in 5 min, whereas with penetration from the receptor surface, full staining required 8–10 min diffusion time. That inhibitors of the ERG work faster when applied to the receptor surface of the retina therefore cannot reasonably be attributed to a diffusion barrier near the vitreous surface.

The rapid effect of ouabain suggests that the structures affected should be easily available to the extracellular solution and should have a very small intracellular volume and a relatively large surface area. Such structures include the synaptic endings of the receptor and particularly the bipolar cells, the ciliary connective between the inner and outer segments of the rods and cones, and the outer segments themselves. The latter, although large, have much of their internal volume occupied by the membranous saccules (discs); and moreover, in places the saccules are continuous with the plasma membrane, greatly increasing its surface area (Cohen, 1963).

Following the application of cardiac glycosides, the b-wave is the first component of the response to disappear. The same is true following application of other inhibitory substances to the isolated frog retina (Hamasaki, 1964). There is evidence that the b-wave originates from the region of the bipolar cells (Brown and Wiesel, 1961; Brown and Watanabe, 1962; Cone, 1963), but if this is true, its disappearance could be caused by a blockage of the transmission path anywhere between the outer segments and the bipolars. PIII, of which the a-wave is presumably the leading edge, may be a receptor potential (Brown and Wiesel, 1961; Brown and Watanabe, 1962). That it is abolished suggests that the glycosides directly affect the rods and cones themselves. It must be considered, however, that several critical sites of action may exist.

*Note Added in Proof*  A recent and alternative view of the a-wave of the ERG is that it arises not in the receptors but perhaps in the horizontal cells and is not causally related to the generation of the b-wave (*cf.* Dowling, J. E. 1967. *Science.* 155:273). A second and more important point (R. N. Lolley, personal communication) has evidence that the ouabain-sensitive ATPase activity of conventional preparations of outer segments is not, in fact, associated with the outer limbs, but with other membranous material.

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