[3H]OUABAIN AUTORADIOGRAPHY OF FROG RETINA

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ABSTRACT

The kinetics and distribution of ouabain binding in retinas of Rana pipiens were examined quantitatively by scintillation counting and freeze-dry autoradiography. The time-course of binding at several concentrations was consistent with a bimolecular reaction. Estimated equilibrium binding levels gave a Michaelis-Menton relationship with a $K_m = 8.3 \times 10^{-8}$ M and a maximum binding level ($B_{max}$) = $4.4 \times 10^{-8}$ mol/g protein. The distribution of binding sites measured autoradiographically varied considerably between layers. The photoreceptor, inner plexiform, and optic nerve fiber layers exhibited the heaviest binding. Within the photoreceptor layer, binding was nonuniform. Binding in the outer segment decreased distally, averaging $\sim 4\%$ of that in the proximal receptor layers ($B_{max} = 4.6 \times 10^{-6}$ M). The origin of the outer segment activity is uncertain at light microscope resolution, as it may be a result of inner segment calyceal processes. Binding within the proximal receptor layers was also nonuniform. Several peaks were observed, with those at the inner segment and synaptic layers being especially noticeable. Assuming an absence of glial cell binding in the proximal receptor layers, we calculated there to be $13 \times 10^6$ ouabain or Na$^+,K^+$ pump sites per rod receptor. Limited measurements suggest a $B_{max}$ of $\sim 8 \times 10^{-6}$ M for the inner plexiform layer.

Measurements of vertebrate photoreceptor currents (19, 44), potentials (3, 7, 41), and Na$^+$-dependent volume changes (23) are the foundation for the widely held view that there is a continuous flow of positive ions out of the proximal receptor, balanced by an equivalent flow of positive ions into the outer segment. The outward current may result from K$^+$ diffusing down its electrochemical gradient and possibly from outward electrogenic pumping of Na$^+$. The outer-segment ionic current is attributed to Na$^+$ diffusion down its electrochemical gradient through membrane channels. The absorption of light by photopigments located in the outer segment causes the release of a transmitterlike substance (15), possibly Ca$^{2+}$ (18), that closes these channels. Consequently, current flow between inner and outer segment is greatest in the dark and is reduced upon exposure to light. Correspondingly, vertebrate photoreceptors (40), unlike their invertebrate counterparts (16), are partially depolarized in the dark and repolarized in light. The ionic gradients supporting these currents are thought to be maintained by Na$^+,K^+$-ATPase pumps, because the currents are abolished by ouabain (14, 42, 44).

An inner-segment location for the Na$^+,K^+$ pump has been suggested by Hagins, Penn, and Yoshikami (20, 42) on the basis of their observations of current flow. If the stoichiometry of the pump is 3 Na$^+/2$ K$^+$ as reported for squid axons (11) and erythrocytes (29), it is electrogenic and a proximal location would contribute approximately...
one-third of the outward current, whereas an outer-segment location would reduce the inward Na⁺ current by approximately one-third as compared to a neutral pump.

Biochemical studies of retinal Na⁺,K⁺-ATPase activity using cell fractionation methods have produced various results. There are claims (6, 13, 21) that outer segments possess a high activity and there are counterclaims (4, 8) suggesting that the reported activities result from contamination with inner segments. Na⁺,K⁺-ATPase activity in other regions of the retina is unknown. Histochemical studies on the distribution of Na⁺,K⁺-ATPase in neural tissues are rare (5, 10, 35).

In this study, we have used quantitative light-microscope autoradiography of [³H]ouabain (37) to locate and measure the number of Na⁺,K⁺ pump sites per photoreceptor and their distribution within the receptor layer.

MATERIALS AND METHODS

Experimental Protocol

_Rana pipiens_ obtained from a local supplier were kept ≤2 wk in a laboratory tank at room temperature. All of the following procedures were carried out under typical laboratory illumination. For the experiments, the animal was decapitated and the head pinned to a corkboard. The lids were cut away and the eye was hemisected at the level of the ora serrata. The retina, with choroid attached, was removed to a dish of 10°C frog Ringer's and dissected into 5-mg wedges. During the dissection, the choroid and most of the pigmented epithelial cells separated from the retina. The wedges were incubated in frog Ringer's containing appropriate concentrations of radiolabeled drugs. After prescribed incubation periods, the tissue was washed in drug-free Ringer's at 0°C for 20-30 min. When autoradiographs were to be prepared, the incubation and wash solutions contained 5% bovine albumin (Matheson, Coleman and Bell, Norwood, Ohio). All solutions were in equilibrium, with 95% O₂ and 5% CO₂.

In the kinetic measurements of ouabain binding using scintillation counting, retina was digested in 0.1 N NaOH overnight and neutralized (HCl) before analysing for total protein (9) and radioactivity (34). [³H]Ouabain and [¹⁴C]inulin (New England Nuclear, Boston, Mass) were found to be 98% radiopure by thin-layer chromatography. Ouabain concentration was measured by UV spectrophotometry.

To determine the extent of extracellular washout and to ensure that bound ouabain was not removed by this procedure, we measured the time-course of ouabain and inulin loss at 4°C. Fig. 1a shows the washout curves of retina incubated 20 min in 4 × 10⁻⁹ M [³H]ouabain and 0.01% [¹⁴C]inulin. Under this condition of low specific activity, the ouabain present is primarily extracellular and its runout curve is nearly coincident with that of inulin. The time-course for inulin washout was somewhat slower than that (t₁/₂ < 2 min) reported by Ames and Nesbett (1). We advance no explanation for this disagreement. Methodology and species difference seem to be trivial. The washout kinetics ob-

![Figure 1](https://example.com/figure1.png)

**Figure 1** Retinal washout kinetics. Ordinates show the fraction of inulin (Δ, ▲) and ouabain (○, ○) remaining after the indicated periods in drug-free medium at 0°C. Open and closed symbols indicate experiments with and without solution bubbling, respectively. (a) Retina was incubated for 20 min in 4 × 10⁻⁹ M [³H]ouabain (4 μCi/ml) and 0.01% [¹⁴C]inulin (1 μCi/ml). (b) Retina was incubated for 20 min in 10⁻¹⁰ M [³H]ouabain (4 μCi/ml) and 0.01% [¹⁴C]inulin (1 μCi/ml).
served here are very similar to those reported by Shaver and Stirling (34) in renal slices (0.5-mm thick).

**Autoradiography**

After the wash period, tissue was processed for autoradiography as previously described (39). Briefly, frozen tissue was freeze-dried at −40°C under a vacuum of 0.001 torr. The dried tissue was placed in a desiccator, fixed with osmium tetroxide vapor overnight, and then infiltrated in Spurr’s low-viscosity embedding medium (Polysciences, Inc., Warrington, Pa.). The tissue blocks were oriented in flat embedding molds and sectioned (1 μm) over water. Sections, on glass slides, were coated with nuclear track emulsion (Kodak NTB 2, Eastman Kodak, Rochester, N. Y.) by dipping. After drying and various exposures, they were developed (Kodak D-19), fixed and, in some instances, stained (31). Chemography tests produced no evidence of spurious grain production. However, apparent latent image fading over the photoreceptor outer segments was observed occasionally in initial experiments. This was attributed to section rippling, which produces uneven emulsion coating in the photoreceptor region, and/or to incomplete drying of the emulsion. In all of the experiments reported, additional changes of the desiccant were carried out to assure complete drying, and fogged (light-exposed) autoradiographs were included to ensure that neither latent image fading nor rippling had occurred.

The absolute ouabain concentration over a region of the retina was determined by comparing the grain density (grains per square micrometer) with that of the incubation medium. In all experiments, several sections of the incubation medium cut from a sample processed for autoradiography were included on the slide. As a check of the quantitative reliability of the method, a series of standards were prepared from albumin-frog Ringer’s containing various concentrations of ouabain and tritium activity. Measurements of these standards are presented in Fig. 2. They show that the grain densities up to 400 grains/1,000 μm² are proportional to the concentration of tritium-labeled ouabain. This standard curve agrees with that published for tritium-labeled galactose and phlorizin (39). For a given level of radioactivity (μCi/ml), grain density also increased linearly with exposure time in the range of 0–400 grains/1,000 μm² (measurements not shown).

**RESULTS**

**Kinetics of Binding**

To determine the extent of binding-site saturation at a given ouabain concentration and incubation time, we systematically examined the dependence of binding on these parameters. Fig. 3 shows that the initial rate of ouabain uptake increases with concentration. As incubation time increases, the rate of uptake slows and approaches a plateau or equilibrium value at the higher concentrations. Presumably, the lower concentration curves would have reached a plateau had longer incubation times been used. They were not used because the morphology began to deteriorate in retina exposed to ouabain >60 min.

![Figure 3](https://example.com/figure3.png)  
**Figure 3** Kinetics of [3H]ouabain binding to whole retina. Pieces from six animals were incubated for the indicated times and concentrations. The number above each curve gives the ouabain concentration in micromoles per liter, and % refers to ouabain bound in 10⁻⁸ mol/g of protein. A gram of protein was equivalent to 20 g of wet retina. [¹⁴C]Inulin, present in the incubation medium, was used to correct for extracellular ouabain after a 30-min wash in 0°C drug-free Ringer’s. Each point is the mean of six measurements. The fractional SD of the fit for a given curve was typically 12%. Error bars were omitted so as not to clutter the diagram.
In a related study (34), ouabain binding to renal tubules exhibited similar properties that were shown to be consistent with a bimolecular reaction. The data of Fig. 3 are also consistent with a bimolecular reaction, as the curves show. They are least squares fit to the bimolecular equation. Details of the method of analysis were published recently in this journal (34). In characterizing glycoside binding, it is desirable to know the maximum binding level (B\textsubscript{max}) and the equilibrium binding constant (K\textsubscript{m}). To obtain these parameters, one needs to know the dependence of ouabain bound at equilibrium (B\textsubscript{e}) on the free concentration ([O]) of ouabain. At the higher concentrations of Fig. 3, the binding reaction is near equilibrium. At the lower concentrations, especially 0.1 and 0.02 x 10\textsuperscript{-6} M, departure from equilibrium is significant. We assumed that binding at all concentrations was bimolecular and estimated the equilibrium values by extrapolating the curves of Fig. 3 to infinite time (34). Fig. 4 shows a plot of these values as a function of concentration. The values fit the familiar Michaelis-Menton relationship rather well: B\textsubscript{e} = B\textsubscript{max}[([O]/([O] + K\textsubscript{m})]), where B\textsubscript{max} = (4.4 ± 5) x 10\textsuperscript{-8} mol/g protein and K\textsubscript{m} = (8.3 ± 1) x 10\textsuperscript{-8} M. These results suggest that ouabain binds to a single high-affinity site of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase with bimolecular kinetics. The absence of a significant linear component of binding, as observed by Mills and colleagues (26) in the frog skin, further suggests that nonspecific binding is negligible.

**Autoradiography**

The distribution of ouabain binding sites in the photoreceptor and inner nuclear layers under saturation conditions is shown in the autoradiograph of Fig. 5. The most striking feature is the difference in grain densities between the outer segment and the remainder of the receptor. An occasional receptor exhibits a region of binding that extends into the outer-segment zone. These may be green rods (arrows) whose inner segments extend beyond the ciliary junctions of the more numerous red rods (27). Within the proximal layers, binding appears to be most dense at the ellipsoid region and the synaptic or outer plexiform layer.

A higher magnification view of receptor binding in another animal is shown in Fig. 6. The difference in grain density between the outer segments and proximal receptor layers is clearly evident. However, the grain density of the first several micrometers of the outer segments is significantly above background levels and appears to decrease distally toward their tips. This impression is supported by grain-density measurements of serial sections (presented in a later section). The grains of the proximal receptor usually are associated with cell boundaries; this is especially evident in the nuclear regions. However, grains are sometimes found over cytoplasmic regions. This probably results from the inclusion of cell membrane in the plane of sectioning, which is to be expected with 1-μm sections of small cells (7-μm Diam) with complex surface folds. The cluster of grains over the proximal receptor are regions of high density as quantitative analyses show (Fig. 11a); they may result from a high cell-membrane surface area in these regions.

Binding density levels decrease sharply as one passes from the receptor layer to the inner nuclear layer (Fig. 5), which is filled principally by cell bodies. As shown in Fig. 7, activity picks up again in the inner plexiform layer, falls again over the ganglion cell bodies, and rises over the optic nerve fibers. Thus, in the nonreceptor layers, the binding activity is the highest in the regions possessing fine processes and synapses.

The specificity of binding was examined by two established methods (30, 36): raising the concen-
FIGURE 5  Low-magnification autoradiograph of retina incubated for 20 min in $2 \times 10^{-5} \text{ M (25}\,\text{Ci/ml)}$ and washed for 20 min. The preservation of morphology is generally quite good. Rod outer segments (ROS) and mitochondria-rich ellipsoids (E) are darkly stained by osmium. Frequently, individual outer segments exhibit variation in osmium staining; its cause is not known. Freezing has produced an occasional fracture (F) and marked striations in the outer segments; the latter are probably related to the incisures observed (32) with conventional fixation. Other characteristic structures are the double row of nuclei in the outer nuclear layer (ON), the outer plexiform and synaptic layers (S), and the inner nuclear layer (IN) with glial fibers (G). Arrows indicate green rods. Exposure, 8 d; bar, 10 μm.

Grain-Density Measurements

The number of Na⁺ pump sites per rod and their distribution is a problem of considerable interest. To answer the question, we applied grain-density analyses to autoradiographs similar to those presented. First, we established that the kinetics of binding to photoreceptors did not differ significantly from those observed for the whole retina. Pieces of retina from a single animal were incubated in high specific activity [3H]ouabain at the concentrations and for the times shown in Fig. 10 (see legend for experimental details). The curves were obtained as in Fig. 3. These experiments demonstrate that the kinetics of binding to the receptor layer as measured autoradiographically differ little from those measured in the whole retina by scintillation counting. The initial binding rates are slightly faster in the receptor layer. This is not surprising, because it is more accessible than the interior layers, particularly the inner plexiform...
layer, which is a major contributor to total retinal binding. The maximum or saturation binding level, estimated from the $10^{-5}$ M curve for this animal, was $5.1 \pm 0.6$ SE. Maximum photoreceptor binding levels in four additional animals averaged $4.6 \pm 0.09$ SE. The maximum binding level of the receptor layer is approximately twice that measured in retinal slices, which is reasonable, considering the nonuniformity of binding between layers (Fig. 5).

Receptor Distribution

The measurements shown in Fig. 10 represent the mean binding level for the proximal half of

1 $20 \text{ g of wet retina was equivalent to 1 g of protein. Assuming a retinal density of 1 g/ml, then } 10^{-8} \text{ mol/g of protein is equivalent to } 5 \times 10^{-5} \text{ M. Thus, a } B_{\text{max}} \text{ of 4.4 } 
\times 10^{-7} \text{ mol/g of protein (Fig. 3) is equivalent to } 2.2 \times 10^{-5} \text{ M.}$
approximate anatomical location of the measurements. The valleys coincide roughly with the nuclear layers and the last peak coincides with the synaptic layer. Representative autoradiographs from selected locations are shown in Fig. 11b.

**Inner Plexiform Layer**

Autoradiographic measurements of this layer are not complete; however, we present preliminary experiments in Fig. 12 that indicate the maximum level of binding and a problem encountered in measuring the kinetics of binding. At $10^{-5}$ M, binding proceeded at a rate slightly slower than that presented in Fig. 3 and approached a plateau value of nearly $8 \times 10^{-6}$ M in 30 min. However, binding at the lower concentrations proceeded at a much slower rate than those in Fig. 3. Binding to the photoreceptor layer at the lower concentrations in these experiments was similar to that in Fig. 10.

This behavior, typical photoreceptor binding accompanied by low inner plexiform layer binding at low doses of ouabain, was noted occasionally in other autoradiographic experiments. In all of these experiments, we noted that the retina had folded, thereby constricting diffusional access to the inner layers via the vitreous pathway. A possible explanation for this phenomenon is that photoreceptor binding at low concentrations significantly reduced the amount of ouabain reaching the more proximal layers, thereby slowing their rate of binding. At higher concentrations ($10^{-5}$ M), the extraction of ouabain along the diffusional pathway is negligible and binding proceeds at its usual rate. Similar penetration problems with ouabain were observed in avian salt-gland slices (12).

**DISCUSSION**

The kinetic analysis presented here shows that the characteristics of ouabain binding to the retina are equivalent to those observed in other cellular systems (2, 33, 34). The presence of a stably bound
fraction in 0°C ouabain-free medium (Fig. 1) is consistent with a highly temperature-dependent dissociation rate (2, 33, 34). The high binding affinity ($K_m = 8.3 \times 10^{-6}$ M) for retinal pump sites agrees with the observations of Frank and Goldsmith (14) on ouabain inhibition of frog retinal Na$^+$,K$^+$-ATPase ($K_i = 10^{-7}$ M) and those of Mills and colleagues (26) on ouabain binding to frog skin. Furthermore, this agreement shows that extrapolation to equilibrium binding is a reasonable method of estimating the equilibrium binding constant of cardioglycosides in intact tissues. It should be noted that this method is only valid when diffusion rates are fast compared to the association rate (34) and when competing reactions do not limit access to the site of interest (Fig. 12).

The absence of a significant linear uptake component (Fig. 4), the reduction of binding in high K$^+$ medium (Fig. 8), and the apparent absence of binding at low specific activities (Fig. 9) taken together leave little doubt that nonspecific binding is negligible.

We did not examine the relationship between ouabain concentration and retinal response to light as an additional test of specificity, as this was done by Frank and Goldsmith (14). They observed ~50% inhibition of the electroretinogram (ERG) $b$ wave at $10^{-9}$ M and 30 min of exposure (14, Fig. 2), whereas we observed 60% saturation of binding sites at $0.8 \times 10^{-6}$ M and 30 min of exposure (Fig. 3). A slight delay between binding and inhibition of the ERG is to be expected, because the ionic gradients must run down for the latter to occur.

Receptor Pump Site Distribution

The major problem that this study set out to solve was how Na$^+$,K$^+$ pump sites are quantitatively distributed in the photoreceptor layer. The
results in Fig. 11 answer this question at the micrometer level. Clearly, there are many more (>90%) pump sites in the proximal layers than in the rod outer segments. It is also equally clear that the modest outer segment binding is specific, for no such binding was observed under high K+ and with high glycoside concentrations.

The origin of the outer segment sites is certainly a question of interest. Because of light microscope resolution limits, the possibility cannot be excluded that ouabain binds to the fine calyceal processes of the inner segment that extend a considerable distance within the incisures of the outer segment. Preliminary [3H]ouabain autoradiography at the electron microscope level (5) indicates that bullfrog calyces bind ouabain. If, on the other hand, some of the binding sites are located on the outer segment membrane, did they arrive there by diffusion or by a directed process, as in the case of rhodopsin (42)? That binding is most dense at the ciliary junction and declines to zero near the tip suggests diffusion. However, membrane protein diffusion rates (24, 28) are much too rapid compared to outer-segment renewal time (6 wk, 43) to account for the marked gradient of binding shown in Fig. 11. Whatever their origin, their presence could account for the reports (6, 13, 21) of Na+,K+-ATPase activity in isolated outer segments.

These observations raise yet another question of basic interest: how the photoreceptor directs Na+,K+-ATPase to the proximal layer and rhodopsin to the outer segment. This problem is related to that of epithelia where, for example, in the small intestine Na+,K+-ATPase is directed to the basolateral membrane (36), whereas sugar transport sites are directed to the microvillar membranes (39). In the latter case, the tight junction may segregate these two kinds of transport sites. It is not clear how this segregation is effected in photoreceptors.

It is also apparent from Figs. 5 and 10 that Na+,K+ pump sites are not distributed uniformly within the proximal half of the receptor layer, as several peaks and valleys of activity were observed. The results in Fig. 11 should be interpreted with caution, as all regions of the retina may not show this precise grain-density distribution. The measurements were derived from a population of ~60 adjacent cells and may be peculiar to the region of the retina analyzed. Nevertheless, we invariably observed a sharp rise in activity at the ciliary junction and an abrupt decline beyond the outer plexiform layer. A major point to be garnered from this analysis is that the pockets of high activity seen in the proximal receptor are real and not the result of random variations in grain density. They may be a consequence of an increase in specific density (number per square micrometer of membrane) or simply a local increase in membrane area. The answer awaits a higher-resolution, quantitative method of localizing these pump sites.

**Na+ Pump Site Density and Cycle Rate**

Assuming that each pump site or enzyme binds one molecule of cardioglycoside and that nonreceptor binding in this layer is negligible, the number of pumps per rod is given by Avogadro's number times the ratio of the maximum binding concentration (4.6 x 10^-6 M) to the volume of the proximal half of a single receptor (~4.700 μm^3). This calculation yields the value of 13 x 10^6 sites/rod, especially impressive when compared with the human erythrocyte, which possesses ~200 pump sites. Comparable densities have been reported for epithelial cells (22, 26, 30, 34, 37), which also produce large active Na+ fluxes.

From the molecular point of view, it is desirable to know the cycling rate of the pump. Zuckerman (44) has estimated from current measurements that about 1.5 x 10^9 Na+ ions flow into a frog rod outer segment each second. If, as in the erythrocyte (29) and squid axon (11), three Na+ ions are pumped out and two K+ ions are pumped in per cycle, then the cycle rate for an inner-segment pump would be about 4/s. This value agrees reasonably well with calculations (unpublished observations from
FIGURE 11a Autoradiographic analysis of binding within the photoreceptor layer of retina incubated for 20 min in $10^{-5}$ M $[^{3}H]$ouabain and washed for 30 min. Grain density ($G$) is in grains/10$^3$ $\mu$m$^2$; distance ($\mu$m) is in micrometers. The symbols in the receptor diagram are as in Fig. 5; C, calyceal process. Each point is the mean of five measurements. Except at selected points, SE bars were omitted so as not to clutter the graph.

this laboratory) for other frog cells of 9/s in sartorius muscle and 77/s in skin epithelial cells. A slightly higher cycle rate (27/s) is obtained if the sodium flux estimate ($10^9$ ions/s $\times$ rod) of Korenbrot and Cone (23) is used. In the above calculations the small contribution of cones to the receptor layer has been ignored.

Other Layers

Both the inner plexiform and optic nerve layers exhibited high binding activities. Measurements were not made of the latter. The preliminary data of Fig. 12 suggest that the pump site content of this layer is nearly twice that of the receptor layer. Observations on the retinal epithelium were not presented, as these were ambiguous. In several instances, clumps of the retinal epithelial cells remained loosely attached to the outer-segment layer. We occasionally saw a low level of binding to the apical surface; however, in other experi-
ments we did not. Access to the apical surface was not a problem, as the adjacent receptor layer exhibited heavy binding. Silver grain detection at the light microscope level is complicated by the presence of pigmented granules of similar size; hence, we did not pursue this question further. One would expect an apical site of binding from recent studies by Miller et al. (25), and a preliminary report (5) of apical binding was recently published.

Finally, it should be noted that proximal to the outer limiting membrane, the cell type responsible for binding cannot be specified because of resolution limitations. It is doubtful that extension of the present technique to the electron microscope level (5) would answer this question. Perhaps his-
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