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Light-Enhanced Inhibition of Ouabain Binding to Digitalis Receptor in Rat Brain and Guinea Pig Heart by the Food Dye Erythrosine

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SUMMARY

Erythrosine (ERY) (FD & C red no. 3) inhibited specific binding of [^{3}H]ouabain to rat brain homogenates with an IC_{50} of 23 μM in the dark and 1 μM in ordinary fluorescent light. Competition studies demonstrated the presence of two components, only one of which was affected by light. Lineweaver-Burk analysis indicated that ERY preferentially antagonizes [^{3}H]ouabain binding at a high-affinity site in the light, whereas in the dark the dye inhibits binding in a manner qualitatively similar to inhibition by ouabain. Light enhancement of ERY potency occurred only when dye and tissue were present together in the incubation medium, pointing to participation of transient molecular species. However, neither superoxide dismutase nor catalase altered the effects of ERY in the light or dark, suggesting the absence of oxygen free radicals. When oxygen levels were raised, there was enhancement of inhibition by ERY at a high-affinity receptor accompanied by disappearance of [^{3}H]ouabain binding at one of lower affinity. In contrast to brain, membranes from guinea pig heart showed only one binding site for [^{3}H]ouabain, and antagonism by ERY at this site was markedly enhanced by light. Structural differences between classes of ouabain binding regions probably accounts for the discrimination exhibited by ERY in the presence of light and oxygen. Our findings also caution that metabolic transformation of this common food dye, light decomposition, or photoreaction with foodstuff may yield more toxic derivatives.

INTRODUCTION

Since early reports linking the use of artificial food colors with hyperactivity, learning disabilities, and otherwise undesirable behavior in humans, numerous studies aimed at substantiating these claims under controlled conditions have been undertaken (1–10). In general, results from human studies have been controversial. Moreover, attempts to correlate clinical or behavioral findings with specific cellular mechanisms from *in vitro* studies have not met with much success.

Growing attention has been directed toward the xanthene dye ERY[2] (Fig. 1) and its continued use in food, drugs, and cosmetics. ERY is reported to increase the frequency of miniature end-plate potentials at frog neuromuscular synapses (11), uncompetitively inhibit dopamine transport in synaptosomes derived from rat caudate nucleus (12), and elevate resting membrane potential and conductance in isolated mollusk ganglia (13). Logan and Swanson (14) found that ERY alone, among seven commercially used food dyes, inhibited the accumulation of eight different neurotransmitters or neurotransmitter precursors by rat brain homogenate. Silbergeld (15) showed that ERY specifically inhibits a high-affinity component of [^{3}H]ouabain binding in rat brain and inhibits the sodium pump in synaptosomes. Bihler *et al.* (16) reported that ERY, like ouabain, at concentrations as low as 0.1 nM interfered with ion flux and produced positive inotropy in whole perfused guinea pig heart. We have found that ERY, but not other common food dyes, inhibited binding of [^{3}H]ouabain but not [^{3}H]naloxone, [^{3}H]spiroperidol, or [^{3}H]quinuclidinyl benzilate to membranes from rat brain.[3]

We have confirmed the findings of Silbergeld (15) and observed furthermore that light enhances the potency of the dye in the ouabain RRA in both guinea pig heart and rat brain homogenates. Additionally, we show that in the presence of oxygen the potency increase of the dye at a high-affinity ouabain site in rat brain is accompanied by abolition of [^{3}H]ouabain binding at one of lower affinity.

MATERIALS AND METHODS

Reagents. Disodium ATP, erythrosine, ouabain octahydrate, Tris-phosphate, catalase, and superoxide dismutase were purchased from Sigma Chemical Company

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[1] Career Investigator of the Medical Research Council of Canada.

[2] The abbreviations used are: ERY, erythrosine (FD & C red no. 3; 2′,4′,5′,7′-tetraiodofluorescein); RRA, radioreceptor assay.

[3] M. Hnatowich and F. S. LaBella, unpublished observations.

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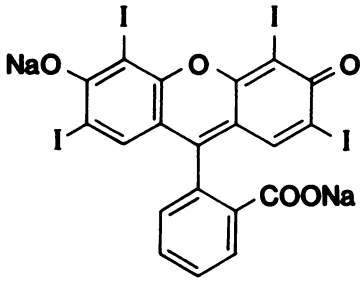

FIG. 1. *Structure of erythrosine*

(St. Louis, Mo.). [^{3}H]Ouabain (10–20 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). All other chemicals were supplied by Fisher Scientific Company (Pittsburgh, Pa.).

[^{3}H]Ouabain RRA. Incubation was carried out exclusively in 45 mM Tris/5 mM $MgCl_2$/5 mM Tris-phosphate, pH 7.4 at 37°. Male Sprague-Dawley rats (200–300 g) were decapitated and their brains were quickly removed, weighed, and homogenized in 20 volumes of ice-cold buffer with a Brinkmann Polytron (Setting 6 for 10 sec). Male or female guinea pigs (300–500 g) were killed by cervical dislocation and their hearts were quickly removed and immersed in buffer (22°) until beating ceased. Atria were discarded and ventricles were weighed, coarsely minced with scissors, and homogenized as for brain. Homogenates were centrifuged once at 35,000 × *g* for 25 min in a Sorvall RC2-B refrigerated centrifuge (0–4°) and the supernatants were discarded. Pellets were resuspended in ice-cold buffer (Polytron Setting 6 for 5 sec) and dispensed into glass culture tubes (12 × 75 mm). Assay mixtures (1.0 ml final volume) consisted of 2.0 nM [^{3}H]ouabain; 10 mg (wet weight equivalent) of tissue (approximately 0.5 mg of protein); and 0–10 μM ouabain (brain), 0–1 μM ouabain (heart), or 0–420 μM erythrosine (brain and heart). Nonspecific binding was determined in the presence of 100 μM ouabain (brain and heart) and never exceeded 5% of the total counts bound. The reaction was initiated by the addition of the tissue suspension, followed by incubation at 37° for 90 min, and terminated by centrifugation at 3000 × *g* for 25 min at 4°. Supernatants were discarded by aspiration and the pellets were dissolved in 0.3 ml of 2 N KOH with heating (70°) for 10 min. Aliquots (0.2 ml) of the membrane digests were added to glass vials containing 10 ml of scintillation medium (156 ml of ScintiPrep 2, 1300 ml of ethylene glycol monomethyl ether, and 2450 ml of toluene) and counted in a Beckman LS 8100 liquid scintillation analyzer. Quenching occurred at concentrations of ERY above 100 μM, and an appropriate correction was applied. Assay tubes were routinely exposed to 15 or 20 min of "normal" fluorescent room lighting (approximately 5 lux) prior to the start of the reaction. After the addition of tissue, assay tubes were either wrapped in aluminum foil [ERY (dark)] or exposed to an additional 65 lux of fluorescent light during subsequent incubation [ERY (light)].

Lineweaver-Burk analyses. RRA tubes contained 1.0 mg of brain tissue per milliliter and varying concentrations of [^{3}H]ouabain (1–80 nM) at fixed concentrations of ERY (0–10 μM), or unlabeled ouabain (0–160 nM). Data

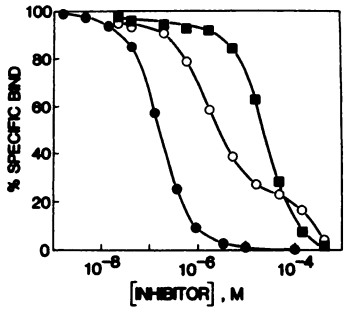

FIG. 2. *Inhibition of specific [^{3}H]ouabain binding to rat brain homogenate by ERY in light and dark*

●, Ouabain; ○, ERY (light); ■, ERY (dark). Duplicate assays (one covered with aluminum foil) were run simultaneously. Values for displacement of [^{3}H]ouabain by unlabeled ouabain in light and dark were identical. Data shown are means of triplicates of a representative experiment. Variability among five experiments was about 3%.

were fitted (when appropriate) to a double-reciprocal, weighted least-squares regression line by a computer program developed in this laboratory. The weights used were the reciprocal variances of complementary bound and free values, whichever variance was larger.

RESULTS

Figure 2 illustrates the light-enhanced inhibition of specific [^{3}H]ouabain binding to rat brain homogenate by ERY. Typically, displacement curves obtained in the light yielded IC_{50} values approximately one order of magnitude less than those obtained in the dark and displayed two components. When exposure time to light was varied during incubation, deviation from the sigmoid profile of either ouabain or ERY (dark) was accompanied by a corresponding increase in potency. In contrast, [^{3}H] ouabain binding to guinea pig heart membranes in the presence of ERY (Fig. 3) showed marked light enhancement of inhibition by the dye but there was no apparent evidence of multiple binding components. Lineweaver-Burk plots of [^{3}H]ouabain binding to brain homogenate in the presence of ouabain and ERY (dark) (Figs. 4 and 5) exhibited patterns characteristic of competitive and linear mixed-type inhibition, respectively. In the light, however, double-reciprocal plots of ERY-inhibited [^{3}H] ouabain binding showed downward curvature near the

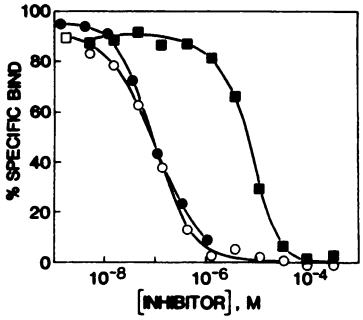

FIG. 3. *Inhibition of specific [^{3}H]ouabain binding to guinea pig heart homogenate by ERY in light and dark*

●, Ouabain; ○, ERY (light); ■, ERY (dark). Details were as described under Materials and Methods and Fig. 2.

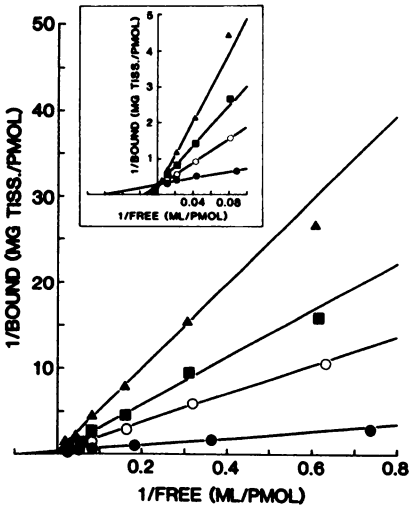

FIG. 4. *Lineweaver-Burk plot of ouabain-inhibited [^{3}H]ouabain binding to rat brain homogenate*

●, Uninhibited; ○, 40 nM ouabain; ■, 80 nM ouabain; ▲, 160 nM ouabain. Data shown are means of triplicates of a representative experiment conducted under "normal" room lighting conditions (approximately 5 lux). There was no significant difference in results obtained from assay tubes wrapped in aluminum foil and those exposed to approximately 70 lux of fluorescent light. Variability among experiments was about 15%. The *inset* shows a scale expansion near the origin.

origin (Fig. 6), becoming more pronounced as the concentration of ERY was increased. This observation is consistent with the finding of Silbergeld (15) that 100 nM ERY completely abolishes [^{3}H]ouabain binding at a high-affinity site in rat cortex homogenate. Figure 7 shows the results of a binding experiment covering a much wider concentration range of [^{3}H]ouabain (0–400 nM in contrast to 0–80 nM). Clearly, the two saturable [^{3}H]ouabain binding components, as demonstrated by Silbergeld (15), are evident. However, we estimate approximately 1200 times the number of sites, albeit the ratio of high- to low-affinity sites is essentially the same. Scatchard transformation of the data summarized in Fig. 7 (not shown)

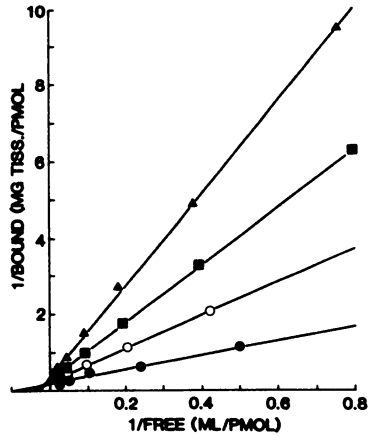

FIG. 5. *Lineweaver-Burk plot of ERY-inhibited [^{3}H]ouabain binding to rat brain homogenate in dark*

●, Uninhibited; ○, 3 μM ERY; ■, 6 μM ERY; ▲, 10 μM ERY. Assay tubes were wrapped in aluminum foil after the addition of tissue. Data are means of triplicates of a typical experiment. Variability among three experiments was about 15%.

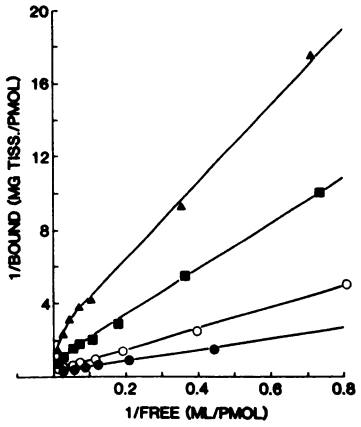

FIG. 6. *Lineweaver-Burk plot of ERY-inhibited [^{3}H]ouabain binding to rat brain homogenate in light*

●, Uninhibited; ○, 15 nM ERY; ■, 45 nM ERY; ▲, 75 nM ERY. Assay tubes were exposed to an additional 65 lux of fluorescent light over "normal" room lighting (approximately 5 lux). Data are means of triplicates of a representative experiment. Variability among four experiments was about 10%.

gives K_D values for the high- and low-affinity sites of about 15 nM and 90 nM, respectively, in good agreement with Silbergeld's estimates. These values are not sufficiently dissimilar to permit the resolution of multiple binding components in [^{3}H]ouabain displacement curves (Fig. 2).

To account for the increased and discriminating inhibitory properties of ERY in brain and heart, experiments were conducted to determine whether stable degradation products or short-lived species were responsible for the observed potency enhancement associated with light exposure. Figure 8 shows the effect of irradiating solutions of ERY overnight at 37° (controls were covered with aluminum foil) followed by assays in the light and dark. No apparent change in relative potency was observed when a previously irradiated sample of ERY was incubated with brain tissue. This finding argues against stable degradation products as specific inhibitors of the high-affinity [^{3}H]ouabain site but, rather, implicates transient molecular species. Catalase or superoxide dismutase, added to the RRA medium in the presence of light (Fig. 9), had no discernible effect on light-induced enhancement of ERY potency; this finding contraindicates the involvement of oxygen free radicals.

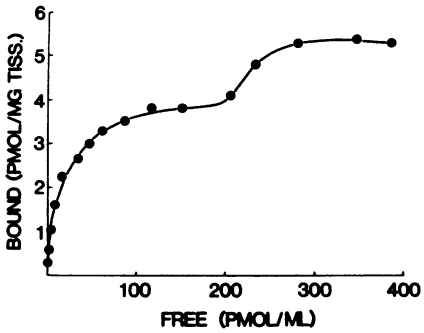

FIG. 7. *Saturation binding of [^{3}H]ouabain to rat brain homogenate*

Assay conditions were as described for Lineweaver-Burk analyses under Materials and Methods. Data are means of triplicates.

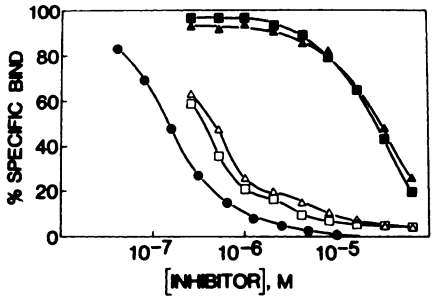

FIG. 8. *Effect of prolonged irradiation of ERY on displacement of specific [^{3}H]ouabain binding to rat brain homogenate in light and dark*

Assay tubes containing identical stock solutions of ERY (one tube was wrapped in aluminum foil) were incubated for 22 hr at 37° under 65 lux of fluorescent light; the solutions were subsequently assayed in the light and dark. Conditions of the assay were as described in Fig. 2. ●, ouabain (nonirradiated/light incubation); □, ERY (nonirradiated/light incubation); △, ERY (irradiated/light incubation); ■, ERY (nonirradiated/dark incubation); ▲, ERY (irradiated/dark incubation).

If short-lived intermediates do contribute to light-enhanced potency, a good candidate is ERY itself. This was partially confirmed by the experiment summarized in Fig. 10. Brain tissue was preincubated with [^{3}H]ouabain alone, [^{3}H]ouabain plus ERY, or ERY alone. After 30 min, appropriate reagents were added to complete the assay mixture and the tubes were incubated for 150 min. One set of tubes was left in the dark for 180 min (preincubation plus incubation), one set was UV-irradiated for 10 min during preincubation, and another set was UV-irradiated for 10 min during incubation (earlier work had shown that UV radiation was also effective in producing

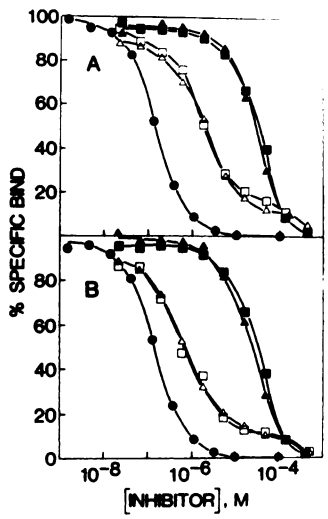

FIG. 9. *Effect of catalase and superoxide dismutase on ERY-inhibited [^{3}H]ouabain binding to rat brain homogenate in light and dark*

A. ●, ouabain; □, ERY plus catalase, 50 μg/ml (light incubation); △, ERY (light incubation); ■, ERY plus catalase, 50 μg/ml (dark incubation); ▲, ERY (dark incubation).

B. As in A except with or without superoxide dismutase, 50 μg/ml. Neither enzyme affected ouabain-inhibited [^{3}H]ouabain binding in the light or dark. The enzymes were present in the assay tubes together with ERY and [^{3}H]ouabain, or ouabain and [^{3}H]ouabain, prior to the addition of brain tissue suspension. Data are means of triplicates.

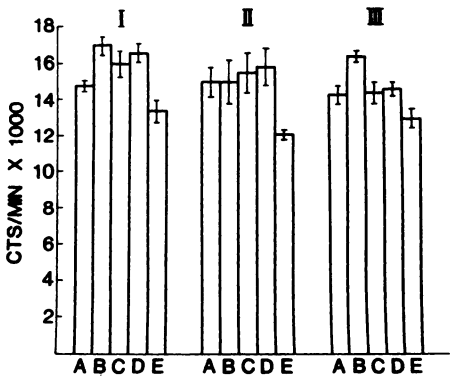

FIG. 10. *Effects of UV irradiation during preincubation and incubation on ERY-inhibited [^{3}H]ouabain binding to rat brain homogenate*

I, No irradation: *A*, tissue alone, [^{3}H]ouabain added at 30 min; *B*, tissue plus [^{3}H]ouabain, buffer added at 30 min; *C*, tissue plus [^{3}H]ouabain, ERY added at 30 min; *D*, tissue plus [^{3}H]ouabain plus ERY, buffer added at 30 min; *E*, tissue plus ERY, [^{3}H]ouabain added at 30 min.

II, 10-min UV irradiation during preincubation.

III, 10-min UV irradiation during incubation.

The UV source consisted of two standard germicidal lamps (General Electric) set at approximately 15 cm from the assay tubes in a closed water bath maintained at 37°. Volumes in all tubes were the same at any given time during the experiment. Manipulations (i.e., reagent additions, covering and uncovering sets of assay tubes with aluminum foil) were done in a darkened room. The ERY concentration was 1.0 μM final (i.e., at 30 min and after). Data shown are means ± standard deviation of triplicates.

enhancement but lengthy exposure caused diminution of binding). In all cases, a significant reduction in [^{3}H]ouabain binding was observed only when ERY and brain homogenate were preincubated together in the absence of [^{3}H]ouabain. This "protection" of the receptor by glycoside provides additional support that a transient form(s) of ERY, with increased potency, is generated during irradiation and specifically interacts with the high-affinity [^{3}H]ouabain site unless the site is already occupied by another ligand.

Results of binding assays on brain tissue carried out in light and dark and under oxygen and nitrogen are shown in Fig. 11. Even greater light enhancement occurred in the presence of oxygen than in air or nitrogen, accompanied by apparent abolition of binding capability at the low-affinity site (and bearing a striking resemblance to the ERY (light) dose-response curve utilizing a guinea pig heart membrane preparation (Fig. 3)). Nitrogen appears to counteract the concerted effect of light and oxygen at the high-affinity site in rat brain.

DISCUSSION

Silbergeld (15) reported the presence of two saturable components of [^{3}H]ouabain binding in rat brain differing in affinity for cardiac glycosides and in sensitivity to erythrosine. Silbergeld observed antagonism by ERY at the high-affinity site only. We have now shown that inhibition by ERY occurs at both sites and is markedly dependent upon oxygen levels and the lighting conditions under which experiments are conducted. In addition, ERY inhibition of [^{3}H]ouabain binding to guinea pig

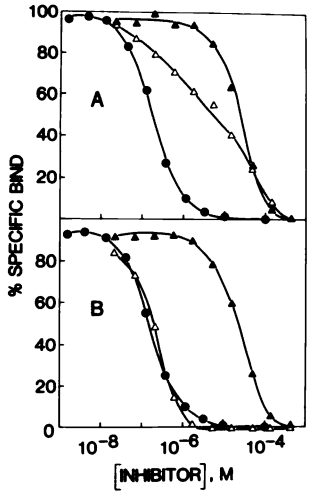

FIG. 11. *Effect of nitrogen and oxygen on ERY-inhibited [^{3}H]ouabain binding to rat brain homogenate in light and dark*

A. Nitrogen: ●, ouabain; ▲, ERY (dark); △, ERY (light).

B. Oxygen: ●, ouabain; ▲, ERY (dark); △, ERY (light).

Duplicate sets of assay tubes were prepared, thoroughly gassed, and corked. Rat brain homogenate was evenly divided between two centrifuge tubes and centrifuged as described under Materials and Methods. Pellets were resuspended, as described, in buffer saturated with oxygen or nitrogen. With the homogenate stirring under an oxygen or nitrogen gas stream, tissue suspension aliquots were dispensed into the assay tubes, which were again gassed under an oxygen or nitrogen stream, corked, and allowed to incubate for 90 min at 37° while either exposed to 70 lux of fluorescent light or wrapped in aluminum foil. Data are means of triplicates.

heart membrane also is enhanced during light exposure, but only one binding component is evident on dose-response curves.

The results summarized in Fig. 7 show a major discrepancy between our work and Silbergeld's (15) in calculated brain receptor density. This is probably due to the fact that our buffer was a Tris-based Mg^{2+} + P_i system whereas that employed by Silbergeld was a Tris-based Mg^{2+} + ATP + Na^+ system. Whittam and Chipperfield (17) have demonstrated that [^{3}H]ouabain binding to brain is optimal in a Mg^{2+} + P_i system, and others have reported ouabain site concentrations between 55 and 75 pmoles/mg of protein in receptors isolated from kidney (18, 19). Therefore, in view of the fact that brain tissue contains the highest levels of (Na^+ + K^+)-ATPase, our estimate of 60 pmoles of high-affinity site per milligram of protein seems neither unreasonable nor essentially incompatible with the results of Silbergeld (15).

Chan (20) showed that photosensitized oxidation by ERY proceeds via a "Type II" mechanism. Following light absorption, ground-state oxygen reacts with an excited triplet state of the dye molecule to form singlet oxygen, which, apparently, oxidizes the substrate. We believe that this mechanism adequately accounts for the observed results from brain homogenates as illustrated by the following. If, in the light, ERY and singlet oxygen act in concert at the high-affinity ouabain site whereas singlet oxygen alone is the principally active antagonist at the low-affinity site, the transient (Fig. 8) and selective (Fig. 11) nature of ERY inhibition and an insensitivity to oxygen free radical scavengers (Fig. 9) would be anticipated. A basis for protection by glycoside (Fig. 10) also is provided assuming that ERY, or an excited form(s) of the dye, actually binds to the high-affinity ouabain site. High-energy species generated upon photon absorption could, prior to decay, react with labile groups favorably oriented in the receptor region. That such moieties can exist is indicated by several reports. Jesaitis and Fortes (21) showed that binding of ATP or ouabain to purified (Na^+ + K^+)-ATPase from *Electrophorus electricus* resulted in protection of one or two reactive groups from attack by fluorescein mercuric acetate. Also, ADP is known to block oxidation of reactive sulfhydryl groups on (Na^+ + K^+)-ATPase (22–24). Thus, light-enhanced inhibition of ouabain binding by ERY at the high-affinity site may be due to the specific nature of reactive groups at that site.

Sweadner's work (25) provides a basis for our interpretation that the high- and low-affinity sites are structurally dissimilar. Sweadner reported two presumably genetically distinct forms of (Na^+ + K^+)-ATPase in brain, one associated with glia and the other with axon membrane. We found light-enhanced inhibition of [^{3}H]ouabain binding by ERY in heart even more pronounced than that in brain, but only one site was apparent in heart (Fig. 3). Although it is tempting to speculate that the ERY (light)-sensitive forms of ouabain receptor from brain and heart are one and the same, we found that the affinity of glycoside binding is markedly different in these tissues under identical assay conditions. Also, Sweadner (25) designated as α the enzyme common to brain and other organs (including heart) and as α(+) the species possessing the greater affinity for [^{3}H]ouabain and being unique to brain. However, we found that the ERY (light)-sensitive form is that of higher affinity and is present in both brain and heart. Clearly, careful purification of receptor from both tissues is required to resolve this apparent anomaly.

The results presented indicate that, under mild *in vitro* conditions, a reactive form(s) of ERY can be generated and shown to interact specifically with ouabain binding sites in both heart and brain tissue. The dye's additional ability to promote singlet oxygen formation (20) and the established consequences thereof (20, 26) cautions for the careful re-evaluation of this and related compounds in their continued use in food, drugs, and cosmetics. The possibility of metabolic transformation of this dye to even more undersirable entities or the products of its photoreaction with foodstuff on the market shelf cannot be overlooked.

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