

Short-circuiting the visual cycle with retinotoxic aromatic amines

(vitamin A/retinoids/rhodopsin/isomerization/phenacetin)

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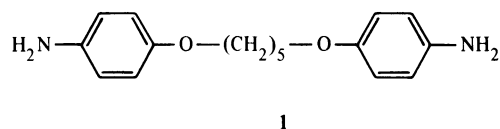
ABSTRACT The retinotoxic drug 1,5-di-(*p*-aminophenoxy)pentane inhibits the accumulation of all 11-*cis*-retinoids in the eye and can deplete preformed stores of them. It is shown here that these effects are not specific to 1,5-di-(*p*-aminophenoxy)pentane but are shared generally by primary aromatic amines containing a hydrophobic tail. Furthermore, certain clinically used drugs, such as the anti-inflammatory drug phenacetin, can be metabolized to produce these retinotoxic amines. It is likely that hydrophobic aromatic amines will in general be retinotoxic, and drugs based on these structures need to be reassessed in this light. It is proposed here that these amines function by catalyzing the isomerization of 11-*cis*-retinal thermodynamically downhill to form its all-*trans*-congener. This mechanism accounts for the lack of structural specificity observed with these compounds and is supported by experimental evidence presented here. Schiff bases formed between 11-*cis*-retinal and a relevant aromatic amine in phosphatidylcholine-based liposomes lead to the formation of the all-*trans* isomer, at rates ≈ 15 times faster than the rate of 11-*cis*-retinal isomerization by itself in these liposomes and 10^2 - 10^3 times faster than the rate of isomerization of this molecule in *n*-heptane. The rates of the amine-catalyzed isomerization are fast enough to account for their *in vivo* effect.

The initial event in vertebrate vision involves the photochemical isomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to its all-*trans* congener (1, 2). Rhodopsin, activated in this fashion, catalyzes the exchange of GTP for GDP in a G protein, which in turn activates a phosphodiesterase specific for cGMP (3, 4). For vision to proceed, the all-*trans*-retinal released from rhodopsin must be converted back into 11-*cis*-retinal, a thermodynamically uphill process (5). The requirements of visual adaptation are such that immediate isomerization of all-*trans*-retinal to 11-*cis*-retinal does not occur after bleaching. Instead the all-*trans*-retinal is reduced, and the resulting retinol is esterified, chiefly to palmitate esters, and stored as such in the pigmented epithelium (6). The whole process can be reversed by hydrolysis of the esters and reoxidation to a retinal. Somewhere in the visual cycle, an energy-dependent conversion of an all-*trans*-retinoid to an 11-*cis*-retinoid must occur. The energy difference between an all-*trans*-retinoid and its 11-*cis* isomer is ≈ 4 kcal/mol (1 cal = 4.18 J) in *n*-heptane or chloroform (5). Indistinguishable equilibrium mixtures are found in more polar milieus such as ethanol or phosphatidylcholine-based liposomes (unpublished data).

This energy-consuming step must be operating in the dark, because in vertebrates, such as frogs, where there is an approximate 3-fold excess of retinoid over opsin, 11-*cis*-retinol esters are generated at the expense of their all-*trans* counterparts in the absence of light (6). Because the enzymes of the visual cycle have not been isolated, and because no useful *in vitro* system is available for study of the visual cycle,

very little is known about it. Critical questions about the visual cycle include the following: (i) What form of retinoid is thermally (dark) isomerized? (ii) Where does the energy come from to drive the cycle and produce 11-*cis*-retinoids? (iii) How is the visual cycle regulated?

One approach to answering some of these questions is to study the biochemical and physiological mechanisms of drugs that affect the functioning of the cycle. From the action of these drugs, important facets of the cycle can be inferred. 1,5-Di-(*p*-aminophenoxy)pentane [1] is the most powerful and specific drug known to affect visual function. From the studies of Goodwin *et al.* in the 1950s it was understood that this drug, originally used as an anti-schistosomal agent, caused night blindness in individuals by inhibiting rhodopsin



regeneration (7). This could eventually lead to long-lasting retinal toxicity. We previously demonstrated that, in addition to blocking visual pigment regeneration, the drug also prevents the synthesis of 11-*cis*-retinal, 11-*cis*-retinol, and 11-*cis*-retinol palmitate (8). Furthermore, the drug actually depletes preformed stores of 11-*cis*-retinol palmitate in the dark (8). The inhibition of regeneration is thus an outcome of the inhibition of synthesis of 11-*cis*-retinoids. Here it is shown that these effects can be brought about by aromatic amines other than 1,5-di-(*p*-aminophenoxy)pentane, and by certain drugs, such as phenacetin, which are metabolizable to primary aromatic amines. In addition, it is shown that these aromatic amines can catalyze the rate of 11-*cis*-retinal isomerization in phosphatidylcholine-based liposomes. These results lead us to suggest that the mechanism of ocular 11-*cis*-retinoid depletion caused by 1,5-di-(*p*-aminophenoxy)pentane and other aromatic amines involves the short-circuiting of the visual cycle by catalyzing the thermodynamically downhill isomerization of 11-*cis*-retinal to its all-*trans* congener.

MATERIALS AND METHODS

Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice.

Chemicals. Aniline, *p*-phenetidine, *p*-*n*-butylaniline, and *p*-*n*-hexyloxyaniline were obtained from Aldrich. Phenacetin and hydroxylamine hydrochloride came from Sigma. Egg phosphatidylcholine was purchased from Avanti Polar Lipids. 11-*cis*-retinal was a gift from Hoffmann-La Roche, and 1,5-di-(*p*-aminophenoxy)pentane (M&B 968a) was generously provided by May & Baker (Dagenham, Essex, U.K.).

Frog Injections. Frogs (*Rana pipiens*, 2.0-2.5 inches) were obtained from Sullivan's Amphibians of North America

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(Nashville, TN) and kept in a cold room until used. All frogs light-adapted overnight 40 cm under a 60 W desk lamp. They were then injected intraperitoneally with an acidified solution of a particular drug in water (10 mg/ml). If the drug did not have sufficient aqueous solubility, absolute ethanol was substituted. Ethanol given to control animals had no effect on either of our *in vivo* assays. Ordinarily, drug dosages were 100 mg/kg unless systemic toxicity necessitated reduced dosage. After injection, frogs remained in the light 1 additional hr before beginning dark adaptation.

Rhodopsin Analysis. Rhodopsin regeneration assays were always performed after 2 hr of dark adaptation. The methods for dissection of the retina and pigmented epithelium and for rhodopsin analysis have been described (8). In some cases, a modified rhodopsin assay was used, which yielded results identical to the original method. This new protocol differed on the following points: (i) The homogenization and detergent buffers contained 15 mM sodium phosphate (pH 6.5) instead of 50 mM hydroxylamine; (ii) 2.5 μ l of 11-*cis*-retinal in isopropanol (5 mg/ml) was used instead of 5 μ l of 11-*cis*-retinal in ethanol (5 mg/ml) for *in vitro* regeneration; (iii) 20 μ l of 2 M hydroxylamine (pH 6.5) was added to the cuvette immediately before the final bleach.

Ester Analysis. Retinol ester analyses were always performed after 24 hr of dark adaptation. The method used has been described (8).

***In Vitro* Isomerization Kinetics.** Individual Schiff bases were preformed (9) by adding 11-*cis*-retinal and a primary amine to egg phosphatidylcholine in chloroform/heptane, and the solvents were removed *in vacuo* for 5 min in a round-bottom flask. A suitable amount of 10 mM Tris-HCl (pH 7.5) was added for a final concentration of 360 μ M phosphatidylcholine, 6 μ M retinal, and 35–85 μ M amine. The suspension was mixed in a Vortex for 1 min, and each flask was incubated in an agitating water bath at 37°C. Time points were taken at 0 time and at 30-min intervals. An aliquot was removed for each time point, and the reaction was quenched with 100 μ l of 1 M NH₂OH (pH 6.5) in a test tube on ice. The retinal oximes were extracted into *n*-heptane, and the geometric isomers were analyzed by HPLC (10). The half-life and first-order rate constant were calculated from plots of the percentage 11-*cis*-*syn*-retinal oxime of the total *syn*-oximes versus time on a semilogarithmic graph.

RESULTS

***In Vivo* Effects of Aromatic Amines.** Initial experiments were performed to determine what modifications could be made in the 1,5-di-(*p*-aminophenoxy)pentane structure while still retaining activity. Since 1,5-di-(*p*-aminophenoxy)pentane is a bifunctional molecule, it was desired to determine whether monofunctional analogs were active. The two criteria used for determining whether an analog was active or not were that the drug should decrease both the rate of rhodopsin regeneration and the rate of 11-*cis*-retinol palmitate formation in the dark in a previously light-adapted animal. In Table 1, it is shown that several different monofunctional aromatic amines gave positive results. In fact, two of them, *p*-*n*-butylaniline [2] and *p*-*n*-hexyloxyaniline [3], were more powerful than 1,5-di-(*p*-aminophenoxy)pentane in their effects on rhodopsin regeneration and on 11-*cis*-retinol palmitate formation. Phenacetin [4], a nonsteroidal anti-inflammatory agent that can be metabolized *in vivo* to form *p*-phenetidine [5] (11), inhibited rhodopsin regeneration but not 11-*cis*-retinol palmitate formation. However, when given in three doses of 100 mg/kg over 24 hr it could inhibit 11-*cis*-retinol palmitate formation. Aliphatic amines such as *n*-butylamine and *n*-octylamine were without effect in our assay systems.

Catalyzed Isomerization of the Retinals by Aromatic Amines. The experiments described above demonstrate a lack of structural selectivity in eliciting the pharmacological

Table 1. *In vivo* effects of aromatic amines on the visual cycle

Aromatic amine	Dosage, mg/kg	% rhodopsin regeneration at 2 hr	% 11- <i>cis</i> -retinol palmitate formation at 24 hr
None	—	97 \pm 2	24 \pm 1
Aniline	100–200	90 \pm 3	23 \pm 2
<i>p</i> -Phenetidine	40–100	60 \pm 8*	7 \pm 4*
1,5-Di-(<i>p</i> -aminophenoxy)-pentane	100–200	46 \pm 5*	12 \pm 5*
<i>p</i> - <i>n</i> -Butylaniline	50–100	15 \pm 2*	3 \pm 1*
<i>p</i> - <i>n</i> -Hexyloxyaniline	60–100	10 \pm 3*	2 \pm 0*
Phenacetin [†]	100	66 \pm 4*	22 \pm 2
Phenacetin [†]	3 \times 100	—	8 \pm 6*

* $P < 0.01$ (one-tailed unpaired *t* test compared to control) for $n = 2-8$. All values are mean \pm SEM.

[†]Phenacetin was dissolved in absolute ethanol. The triple dose was administered as three injections of 100 mg/kg given during a 24-hr period in the light before dark adaptation.

actions of 1,5-di-(*p*-aminophenoxy)pentane and the other aromatic amines. This would suggest that a specific enzyme- or receptor-mediated event is not involved. All that seems to be required structurally is a primary aromatic amine that could insert into lipid bilayer membranes by means of hydrophobic interactions. Since the high-energy 11-*cis*-retinoid pools were run down by the aromatic amines, it is reasonable to consider how these latter agents might affect these pools. The simplest hypotheses that can be developed are that either the drugs enhance the thermodynamically downhill isomerization of an 11-*cis*- to an all-*trans*-retinoid, or they prevent the biosynthesis of an 11-*cis*-retinoid in some manner (8). Very little is known about the latter process, and so whether or not it may be affected is not immediately testable. On the other hand, it is possible to test whether or not 11-*cis*-retinoids are catalytically isomerized by aromatic amines.

The most likely 11-*cis*-retinoid to be isomerized is 11-*cis*-retinal, since it can form a Schiff base with the aromatic amine. To study the possible catalytic ability of aromatic amines to isomerize 11-*cis*-retinal under conditions as close as possible to the physiological situation, egg phosphatidylcholine-based liposomes were prepared containing the Schiff bases of 11-*cis*-retinal and the aromatic amines shown in Table 2. With time, aliquots were removed and the extent of isomerization of the 11-*cis*-retinal was determined by HPLC analysis. As can be seen here, aromatic amines that potently disrupted the visual cycle *in vivo* (Table 1) were potent catalysts in the *in vitro* system. When similar experiments were performed with 11-*cis*-retinol and 11-*cis*-retinol palmi-

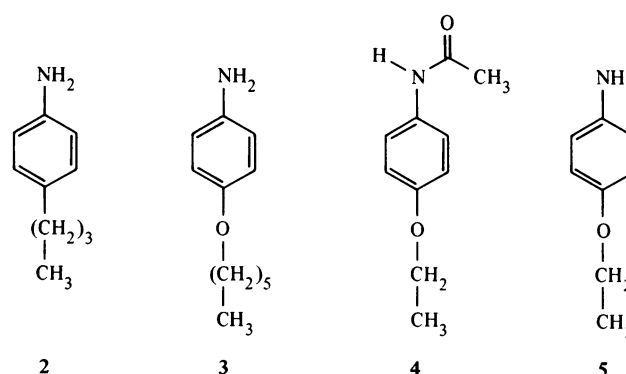


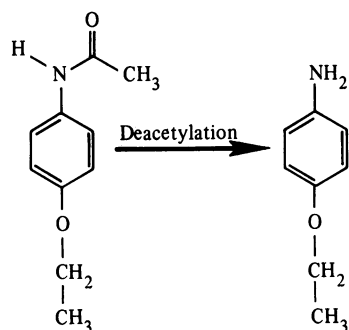
Table 2. Isomerization of 6 μM 11-*cis*-retinal by aromatic amines in 360 μM phosphatidylcholine liposomes at 37°C

Aromatic amine	k , sec^{-1}	$t_{1/2}$, min
None	7.7×10^{-6}	1500
Aniline (85 μM)	2.5×10^{-5}	460
<i>p</i> -Phenetidine (35 μM)	3.1×10^{-5}	370
1,5-Di-(<i>p</i> -aminophenoxy)pentane (75 μM)	1.4×10^{-4}	83
<i>p</i> - <i>n</i> -Butylaniline (75 μM)	1.2×10^{-4}	96
<i>p</i> - <i>n</i> -Hexyloxyaniline (75 μM)	1.2×10^{-4}	96

tate, no isomerization was observed. Phenacetin, which cannot form a Schiff base with retinal without being metabolized first, would not be expected to have any effect in this system. The experiments shown here used an excess of aromatic amine relative to retinal; more recent studies indicate that only ≈ 1 equivalent of amine is required to achieve maximal catalysis (unpublished data).

DISCUSSION

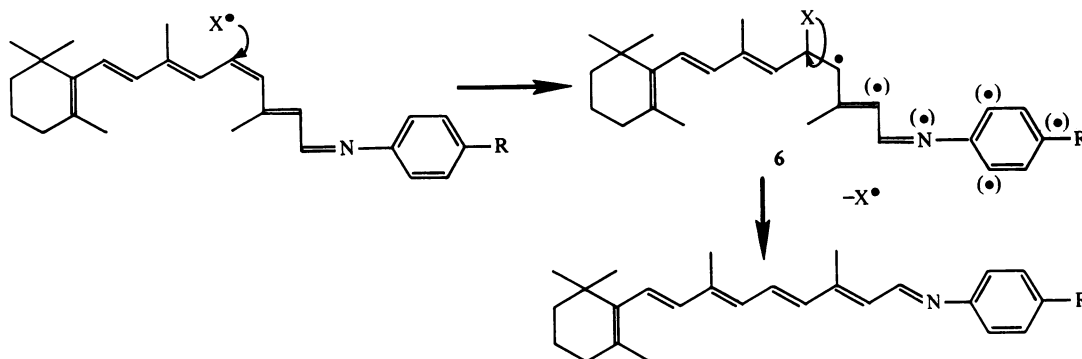
It has been shown here that primary aromatic amines with hydrophobic tails can specifically deplete the levels of 11-*cis*-retinoids in the living eye of the frog. Based on these studies it would be predicted that night blindness and retinotoxicity are properties of a large class of aromatic amines, and the use of these molecules as drugs or as items of commerce should be viewed accordingly. In certain cases, such as with the nonsteroidal anti-inflammatory drug phenacetin, metabolism of a drug can produce a retinotoxic aromatic amine that can cause 11-*cis*-retinoid depletion in frogs (Scheme I). The further use of this drug in humans



Scheme I

needs to be considered in this light.

Since the aromatic amines depleted the stores of 11-*cis*-retinoids, and in so doing showed little structural specificity,



Scheme II

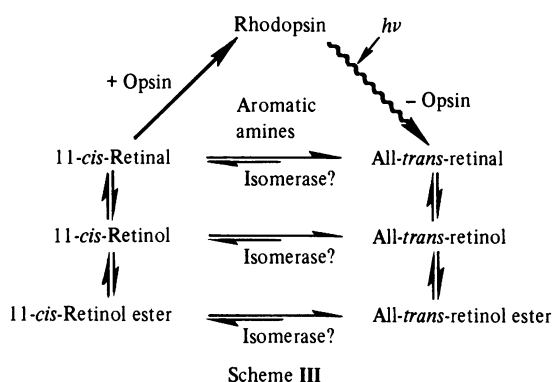
it was reasonable to postulate that they catalyzed the thermodynamically downhill isomerization of an 11-*cis*-retinoid to its all-*trans*-congener. The aromatic amines were without effect on 11-*cis*-retinol or its palmitate ester but did markedly catalyze the isomerization of 11-*cis*-retinal in phosphatidylcholine-based liposomes. Since the aromatic amines catalyze the isomerization of the retinals but not other retinoids, it is reasonable to assume that Schiff base formation precedes the isomerization step. Indeed, Schiff base formation would stabilize the transition state for an isomerization process, given the extended conjugated system produced (Scheme II). For purposes of exposition, a free-radical initiated isomerization process is shown in Scheme II, with attack at C-11. Attack at carbons 5, 7, or 9 would achieve the same result. It is clear that the attack of X^\bullet produces a highly stabilized product [6] that could undergo isomerization followed by the expulsion of X^\bullet to produce the all-*trans*-retinal Schiff base, which could itself hydrolyze to produce all-*trans*-retinal. Among other possibilities, a comparable mechanism in which a nucleophile would be involved in a reversible Michael reaction at C-11 could lead to isomerization. Studies are currently under way in this laboratory to determine the precise isomerization mechanism.

Two further pieces of evidence bear on this proposed mechanism. First, we have now actually isolated Schiff bases from the retina/pigmented epithelium of frogs after intraperitoneal injections of various aromatic amines (unpublished data). Second, Goodwin *et al.* found that *N*-methylated and *N,N*-dimethylated 1,5-di-(*p*-aminophenoxy)pentane analogs were only weakly retinotoxic (7). These results are compatible with predictions based on the proposed mechanism. It should also be noted that the observed *in vitro* rates of isomerization ($\approx 1.3 \times 10^{-4} \text{sec}^{-1}$ or $t_{1/2} = 90$ min) are fast enough to account for the observed *in vivo* results. The half-life of rhodopsin regeneration in the light-adapted frogs is an hour or so, and the half-life of 11-*cis*-retinol palmitate regeneration is measured in days (6). Thus, an isomerization rate of $\approx 1.3 \times 10^{-4} \text{sec}^{-1}$ would abolish the latter process and impair the former, which is what is observed.

The importance of a phospholipid environment, which of course is found in the eye, deserves consideration. At 65°C in *n*-heptane, the thermal rate of isomerization of 11-*cis*-retinal is $2.4 \times 10^{-6} \text{sec}^{-1}$, a $t_{1/2}$ of 80 hr (9). At 37°C, the rate in *n*-heptane would be in the range of 10^{-7}sec^{-1} , while in phosphatidylcholine-based liposomes it is $7.7 \times 10^{-6} \text{sec}^{-1}$. Schiff-base formation with an aromatic amine in these liposomes further increases the isomerization rate 3- to 18-fold (Table 2). By contrast, Schiff-base formation with 11-*cis*-retinal by aniline in *n*-heptane at 65°C has almost no catalytic effect, changing the isomerization rate to just $2.8 \times 10^{-6} \text{sec}^{-1}$ (9). The importance of a phospholipid environment is further supported by the fact that aromatic amines are

progressively more active *in vivo* and *in vitro* as they become more hydrophobic (Tables 1 and 2).

The observation that hydrophobic aromatic amines are potent catalysts of the isomerization of retinoids provides the missing link in the mechanism of action of these drugs. This proposed mechanism uniquely relates the structure-activity relationships of these drugs with their chemical and physiological actions. Therefore, we suggest that these drugs function *in vivo* by short-circuiting the visual cycle and running it downhill thermodynamically (Scheme III).



The consequences of this mechanism of action for the operation of the visual cycle are severalfold. (i) Most obviously, endogenous 11-*cis*-retinal, probably complexed to a binding protein, is susceptible to chemical isomerization *in vivo*. (ii) Many of the enzymes of the cycle must be functional in the dark, or else aromatic amines would not deplete

11-*cis*-retinol esters in dark-adapted animals. (iii) The proposed *in vivo* facilitated isomerization of retinals by aromatic amines argues against the existence of a putative non-energy-coupled retinal isomerase (12), because both should operate indistinguishably *in vivo*. (iv) We have defined a mechanism of ocular toxicity for a family of chemical compounds, and a predictive model of structures likely to be retinotoxic has been provided.

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