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Erythrosine (Red No. 3) and Its Nonspecific Biochemical Actions: What Relation to Behavioral Changes?

Abstract. *Biochemical studies have shown that the ability of erythrosine to inhibit dopamine uptake into brain synaptosomal preparations is dependent on the concentration of tissue present in the assay mixture. Thus, the finding that erythrosine inhibits its dopamine uptake (which, if true, would provide a plausible explanation of the Feingold hypothesis of childhood hyperactivity) may simply be an artifact that results from nonspecific interactions with brain membranes. In addition, although erythrosine given parenterally (50 milligrams per kilogram) did not alter locomotor activity of control or 6-hydroxydopamine-treated rats, erythrosine (50 to 300 milligrams per kilogram) attenuated the effect of punishment in a "conflict" paradigm.*

The prevalence of hyperkinesis and learning disabilities among children, and the fact that therapy with stimulant drugs is not entirely satisfactory (*1*) have made the search for alternative forms of treatment for these problems an important issue. Several years ago, Feingold (*2*) reported that removal of synthetic food colors and other ingredients from the diet of hyperactive children could dramatically eliminate the symptoms in about half of them. The impressive results reported by advocates of this treatment have been evaluated by many groups with the general finding that open studies support Feingold's contentions, whereas closed clinical trials and blind challenge studies have been essentially negative (*3*). Little attention has been given to animal or biochemical studies that might define possible mechanisms underlying the proposed behavioral toxicity of the food dyes. The difficulty in selecting a suitable model for study has been one reason for neglecting this area of research.

Recently, Logan and Swanson (*4*) reported that the mixture of eight food dyes commonly used in pediatric clinical studies caused a significant inhibition of neurotransmitter uptake in homogenates of rat brain. In examining this finding further, they reported that one of the dyes in the mixture, erythrosine (Erythrosin B; FD & C Red No. 3) was responsible for this effect, and that it alone inhibited the uptake of all the transmitters tested. The other dyes were ineffective. Lafferman and Silbergeld (*5*) have confirmed that dopamine uptake into crude synaptosomes is significantly inhibited by erythrosine. The suggestion that one dye might be critical in producing the alleged detrimental effects of the food colors coincided with our interest in hyperkinesis, and prompted us to attempt to replicate these findings and to assess their effect on behavior. We now report that the potency of erythrosine in vitro to inhibit neurotransmitter uptake is dependent on the concentration of tissue present in the incubation system. Thus, the findings of Logan and Swanson (*4*), or Lafferman and Silbergeld (*5*), can be essentially confirmed or refuted depending on the experimental conditions chosen. We also report on behavioral studies in rats that suggest that high doses of erythrosine are active in a paradigm also sensitive to drugs that alter hyperkinetic behavior in children (*6*).

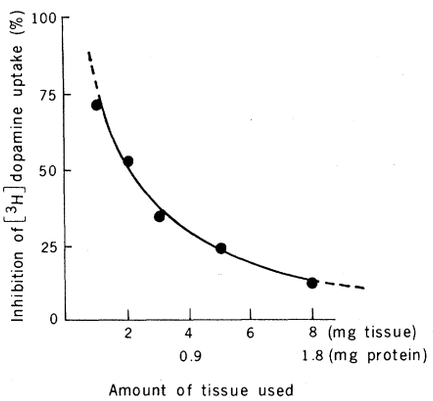

Fig. 1. Ackermann-Potter (*8*) representation of the effects of tissue concentration on [^{3}H]dopamine uptake from a crude synaptosomal preparation from rat striatum. Dopamine uptake was measured essentially by the method of Ferris *et al.* [see (*7*)]. Each data point represents the mean of four separate experiments. The standard error for each data point is never greater than 10 percent of the magnitude of its particular mean.

In our initial experiments (*7*) we failed to confirm the finding reported by others (*4*, *5*) that erythrosine could inhibit the uptake of [^{3}H]norepinephrine or [^{3}H]dopamine into crude synaptosomal preparations of striatum, hypothalamus, or whole brain from Sprague-Dawley rats. In an attempt to resolve this discrepancy, we varied several conditions of the uptake system. These included incubation temperature (0°, 25°, and 37°C), time of incubation with the drug (5, 15, or 30 minutes), presence or absence of light and oxygen, substrate concentration ($10^{-7}M$ and $10^{-8}M$ for norepinephrine, dopamine, or serotonin), erythrosine concentration (0.1 μM to 100 μM), and the concentration of synaptosomal protein present in the medium.

We found that one of the conditions, the concentration of synaptosomal protein present in the incubation medium, influenced significantly the calculated potency of erythrosine as an inhibitor of catecholamine uptake into synaptosomes obtained from all brain areas studied. This is illustrated in Fig. 1, which is a graphical presentation after the suggestions of Ackermann and Potter (*8*) for the case of pseudo-irreversible inhibition. These data clearly show that when the concentration of erythrosine and the concentration of dopamine in the incubation medium are held constant, different degrees of inhibition of dopamine uptake by erythrosine can be achieved by simply varying the concentration of striatal synaptosomal protein present in the medium. The percentage inhibition of dopamine uptake is not directly pro-

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Table 1. Effects of erythrosine on locomotor activity in control and 6-OHDA-treated rats. Erythrosine was dissolved in sterile water (25 mg/ml) and injected into rats (50 mg/kg; the rats weighed 73 to 105 g). Control rats received saline. The 6-OHDA was administered as described previously (*11*). The results are expressed as means (± standard error) for four animals per group.

Treatment	Drug	Locomotor activity	
		Before drug (counts per 60 minutes)	After drug (counts per 20 minutes)
None	Erythrosine	376 ± 73	375 ± 97
None	None (saline)	265 ± 67	190 ± 50
6-OHDA	Erythrosine	438 ± 136	298 ± 92
6-OHDA	None (saline)	302 ± 74	409 ± 139

portional to the concentration of protein present in the medium but, instead, varies in a nonlinear manner with protein concentration.

These results explain the difference noted between Logan and Swanson (*4*), Lafferman and Silbergeld (*5*), and our initial studies. Initially we found only a 12 percent inhibition of dopamine uptake with 100 μM erythrosine when 8 mg of brain tissue was present per milliliter of medium, whereas Logan and Swanson achieved 50 percent inhibition (IC_{50}) at 2 μM erythrosine using 1 mg of whole brain tissue per milliliter of medium. When the concentration of synaptosomal protein was decreased in our studies to 1 mg/ml, greater than 80 percent inhibition of dopamine uptake was achieved with 100 μM erythrosine (Fig. 1), a result consistent with the findings of Logan and Swanson (*4*). Lafferman and Silbergeld (*5*) report an IC_{50} of 45 μM, but do not give the concentration of synaptosomal protein in their incubation system. Our results suggest they used a tissue concentration intermediate between that used in our initial work (8 mg/ml) and that of Logan and Swanson [(*4*), 1 mg/ml]. It is interesting that Lafferman and Silbergeld noted the effects of protein concentration on uptake [see figure legend in (*5*)] but did not investigate it further. These interactions of erythrosine are not confined to effects on dopamine uptake (*9*).

Ultimately, the relevance of any findings in vitro will depend on their correlation with effects in vivo. We therefore examined the effects of erythrosine on gross locomotor activity in rats (*10*) and on the behavior of rats in a conflict (approach and avoidance) test. Newborn rats were injected intracisternally with 6-hydroxydopamine (6-OHDA), a drug that often alters sensitivity to dopaminergic agents (*11*). Control rats of the same age and sex received vehicle only. Both groups of rats were then injected intraperitoneally with erythrosine (50 mg/kg) when they weighed 73 to 105 g (Table 1). The erythrosine had no effect on locomotor activity in either group of rats.

Previous studies of rats in a conflict situation (*12*) have demonstrated that barbiturates and benzodiazepines, which exacerbate the symptoms of hyperkinesis in humans, attenuate the suppressive effect of punishment (that is, increase the number of shocks taken by the animals), whereas amphetamine and other clinically useful drugs will reverse this effect (*6*, *13*). In the present studies, erythrosine significantly increased punished responding in rats given 50 mg/kg or more (Table 2). These doses are much higher than the amounts of a food color mixture that affects learning in hyperkinetic children [about 100 mg administered orally (*14*)]. Only 5 percent of this mixture is erythrosine, so that a 30-kg child would receive an oral dose of about 0.2 mg of erythrosine per kilogram. On this basis alone, the relevance of this finding to the clinical syndrome is questionable. Furthermore, the analgesic effects of erythrosine and its peripheral actions (such as on the kidney) have not been demonstrated, although these effects can contribute to the effects of erythrosine on punished responding (*15*).

We conclude that our data provide a basis for understanding the previously reported (*4*, *5*) effects of erythrosine on neurotransmitter uptake. We hypothesize that erythrosine may have effects on other biochemical processes that result from nonspecific interactions with neural membranes. This is consistent with the conclusion of Levitan (*15*) and explains why Logan and Swanson (*4*) found that erythrosine affects the uptake of many neurotransmitters.

In retrospect, it should have been predictable that this fluorescein derivative would cause unusual effects on membranous systems. Levitan (*16*) demonstrated several membrane interactions of this dye, and speculated about some other possible biological effects. One speculation was that the brain uptake index (a ratio of brain uptake of dye to the uptake of tritiated water 15 seconds after both are injected into the internal carotid artery) should be 82 for erythrosine (*16*). This value, which was not tested experimentally, would mean that significant quantities of the dye should be found in brain. It is known that in rats, approximately 60 percent of oral doses of erythrosine (300 to 600 mg/kg) are passed in the feces (*17*). Moreover, our results suggest that nonspecific interactions throughout the body might dilute the dye sufficiently to prevent effects on specific processes, such as dopamine uptake. Molecular events at the blood-brain barrier, in capillary endothelium for example, would be a more probable site of action than loci deep in the brain parenchyma. Such events might include changes in influx or efflux of amino or organic acids, the influx of organic acids being known to be inhibited by erythrosine in kidney slices (*18*). In any event, information about the pharmacokinetic characteristics of this dye must be obtained before the biochemical data can be properly interpreted.

The data obtained thus far demonstrate that erythrosine does not have specific effects on dopamine uptake and indicate that it is premature to draw conclusions about the relevance of these types of experiments to effects induced

Table 2. Effects of erythrosine and other compounds on punished behavior in rats. See (*14*) for details.

Compound	Dose (mg/kg)	Number of shocks per 3 minutes	Number of animals tested
Sterile water		7.0 ± 0.7	44
Erythrosine	10	8.8 ± 1.5	14
Erythrosine	50	13.0 ± 1.3*	36
Erythrosine	100	15.4 ± 1.7*	20
Erythrosine	300	17.9 ± 2.5*	8
Red No. 40	50	5.3 ± 1.5	8
Chlordiazepoxide	8	20.9 ± 1.8*	11

*Significantly different from sterile water ($P < .001$; Mann-Whitney U test).

by administration in vivo. That high doses of erythrosine affected behavior in a punishment paradigm indicates that further studies on the biological actions of this compound are necessary. Although hyperkinesis is a medical problem, the suggestion that it may be due to synthetic food additives has given it social and political dimensions that increase the need for sound clinical and basic data upon which to make policy judgments. Whatever the outcome of future scientific and clinical experimentation, cautious presentation and interpretation of data will prevent expensive and spurious perturbations of the public and scientific consciousness.

Note added in proof: Locomotor activity was also measured in adult (220 to 290 g) male rats. Activity before drug was 728 ± 65 counts per 60 minutes. Rats (eight in each group) were given erythrosine (100 mg/kg; 50 mg/ml) or saline intraperitoneally. The results were, for the erythrosine-treated rats, 870 ± 146 counts per 120 minutes, and for the saline-treated rats, 326 ± 81 counts per 120 minutes; $.05 < P < .1$, two-tailed t-test.

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Compensatory Increase in Tyrosine Hydroxylase Activity in Rat Brain After Intraventricular Injections of 6-Hydroxydopamine

Abstract. *The neurotoxin 6-hydroxydopamine produced a permanent loss of endogenous norepinephrine and of ^{3}H-labeled norepinephrine uptake sites in the hippocampus within 5 days. These losses were initially accompanied by parallel decreases in tyrosine hydroxylase activity and synaptosomal norepinephrine synthesis. Within 21 days, however, hippocampal tyrosine hydroxylase activity and norepinephrine synthesis rate increased three- to fivefold. These data suggest a novel form of plasticity in brain-damaged animals characterized by an increase in the capacity for transmitter biosynthesis in residual neurons.*

Systemic administration of 6-hydroxydopamine (6-OHDA) destroys most noradrenergic nerve endings in the sympathetic nervous system of the rat. However, the catecholamine-containing chromaffin cells of the adrenal medulla are not destroyed, and within 2 days, the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, is increased in these cells (*1*, *2*). We now report that an analogous process occurs in the central nervous system. The loss of central noradrenergic terminals following the administration of intracerebroventricular 6-OHDA leads to a rapid increase in TH activity in neuronal cell bodies, followed by an increase in TH activity and in norepinephrine (NE) synthesis in residual terminals. These results are discussed in terms of the ability of rats to sustain extensive damage to central catecholaminergic systems with relatively little functional impairment.

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