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14. Learning tests were also given at 2:30 p.m. and 3:30 p.m. For the children who were challenged with 100 mg of food dye blend, a second challenge was administered at 2:00 p.m. This second challenge was not administered to children receiving the 150-mg dose. The interpretation of the data from these last two tests each day is consistent with the results reported here (*23*).
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Behavioral Responses to Artificial Food Colors

Abstract. *Twenty-two young children, maintained on a diet that excluded certain foods, were challenged intermittently with a blend of seven artificial colors in a double-blind trial. Parents' observations provided the criteria of response. One child that responded mildly to the challenge and one that responded dramatically were detected. The latter, a 34-month-old female, showed a significant increase in aversive behaviors. These results further confirm previous controlled studies.*

Feingold (*1*) contends that as many as 50 percent or more of the children labeled as "hyperkinetic" or "hyperactive" can be treated successfully by eliminating from their diet synthetic colors, flavors, and certain fruits and vegetables said to contain "natural salicylates." Feingold's hypothesis emerged from clinical and parental observations, not controlled experimentation, but they were cogent enough to prompt several controlled trials.

We assessed sensitivity to artificial colors in 22 children, 15 male and 7 female, between 2.5 and 7 years old. All were enrolled in the Kaiser-Permanente Health Maintenance Organization (*2*) during the experiment. The problem behaviors of each child had been reported as improved when the child was kept on a diet that excluded artificial colors and flavors for at least the 3-month period preceding the study. None of the participants suffered from clinically significant medical or psychiatric problems; none had been diagnosed as hyperkinetic. To select an appropriate dose of colors, we obtained dietary histories on 80 children who resembled the study population. From this survey, we estimated the mean amounts of seven FD & C certified colors ingested daily, relying on published industry practices as the basis for the calculations (*3*).

The study was conducted as a double-blind trial; each child served as its own control. At a specified time on each of 77 days, the child consumed a bottle of soft drink (*4*) containing either a combination of caramel and cranberry coloring (placebo) or a freeze-dried monoblend of seven colors (Table 1) plus cranberry coloring (challenge). The two drinks were indistinguishable by sight, smell, taste, or stain color. On 8 days distributed randomly among weeks 3 through 10 of the study period, each child received the challenge drink (*5*). No parent or individual member of the study team knew whether a child was being challenged on a given day (*6*).

The children were maintained on a diet that excluded artificial colors and flavors, 14 fruits, 3 vegetables, specified spices and extracts, and the preservatives BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene). Not all parents restricted the designated fruits and vegetables, claiming that their children were not sensitive to these items. We also attached identifying markers to Kaiser membership cards to alert staff to the child's special status because most pediatric drug and vitamin formulations contain artificial colors and flavors.

Parental observations provided our main data. Before a child entered the study, the parent (*7*) sorted a deck of punched cards labeled with items from several standardized behavior inventories (*8*). In successive sorts, the parent narrowed the items to seven aversive behaviors associated with infractions and three positive (typical "good") behaviors. This procedure yielded the ten target behaviors that served as response criteria for each child throughout the 11-week experimental period.

Each day of the study, the parent conducted two 15-minute observation periods, one within 3.5 hours after drink consumption and one at a later time. During these periods, the parent recorded on a form each occurrence of any of the target behaviors. Twenty-four hours after the drink was consumed the parent also recorded a global estimate, on a scale of 1 to 9, of the frequency and severity of each target behavior during that period. The parent also noted any observed or suspected dietary infractions, recorded sleep data, rated the day as a whole, completed the ten-item Conners ques-

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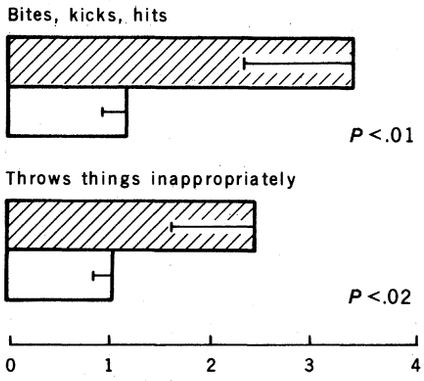

Fig. 1. Mean total day ratings of indicated target behaviors for subject 55. Open bars represent the mean ± standard error of results for days on which the children received placebo; hatched bars represent days on which the children received the challenge.

tionnaire, and commented on any deviations from the typical daily routine. Also, a wrist counter was actuated by the parent each time during the day any of the seven aversive behaviors was observed (*9*). Each weekday the parent was telephoned, interviewed in a partly structured fashion, and reminded to mail the daily data forms. One day a week was randomly assigned for a home visit by a behavioral specialist at a time that coincided with one of the scheduled 15-minute observation periods. The primary aim of these visits was to ensure that the parent maintained stable criteria for the target behaviors. The project nutritionist also visited the parents about once a week.

A clinical trial like ours is not a group experiment, but 22 separate experiments. Our aim was not to estimate population prevalence or sensitivity, but simply to determine if behavioral sensitivity to color additives could be demonstrated in a controlled trial. Accordingly, we analyzed each child's data separately. The primary statistical tool, mainly because of the complex time series structure of the data, consisted of randomization tests (*10*). Parametric techniques generally supported the conclusions derived from the nonparametric methods. Infraction days were excluded from analysis (*11*).

Twenty of the 22 children displayed no convincing evidence of sensitivity to the color challenge. One 3-year-old boy displayed significant elevations in the two target behaviors emphasized as most characteristic of infractions by the mother (Fig. 1). This was largely the contribution of three challenge episodes.

One child reacted dramatically. This 34-month-old girl, weighing about 13 kg, had been eating a diet that excluded colors and flavors, but not the foods classified as natural salicylates, for 6 months. She behaved significantly worse after challenge than after placebo on five of the seven aversive behaviors and on all of the global measures (Fig. 2). One intriguing aspect of this child's response was her mother's ability to discriminate the response to color. She volunteered the information, in the comments section of the daily form, that her daughter had received the challenge six times during the 77-day period. She was correct five times ($P = 1.6 \times 10^{-5}$). These data further strengthen the accumulating evidence from controlled trials (*12*), supplemented by laboratory experiments (*13*), that modest doses of synthetic colors, and perhaps other agents excluded by elimination diets, can provoke disturbed behavior in children. By raising the dose to 100 mg, Swanson and Kinsbourne (*14*) induced adverse effects in 17 of 20 children.

Appraisals of behavioral toxicity are not now a standard, nor even an accepted component of food additive toxicity testing. Yet, the doses employed by us, and most of our fellow investigators, are about 50 times less than the maximum allowable daily intakes (ADI's) recommended by the Food and Drug Administration. ADI's, in turn, typically represent a 100-fold safety factor extrapolated from toxicity testing in rodents. The recent review of food additive safety by a select committee (*15*) foresees behavior as a criterion in future hazard evaluation. We concur.

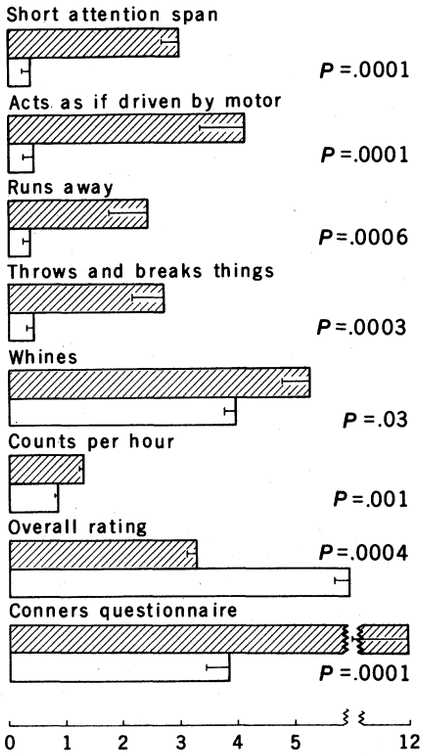

Fig. 2. Mean total day ratings of indicated target behaviors for subject 73 plus corresponding global scores. Open bars represent mean ± standard error of placebo days; hatched bars represent challenge days. The 15-minute tallies recorded about 3 hours after drink consumption were significantly higher on challenge than on control days for behavior 2 ($P = .003$) and behavior 3 ($P = .016$).

Table 1. Composition of the color blend used as the challenge.

Color	Dose (mg/day)
Yellow 5	9.07
Yellow 6	10.70
Red 40	13.80
Red 3	0.57
Blue 1	0.80
Blue 2	0.15
Green 3	0.11
Total	35.26

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5. On occasional days when parents could not comply with the schedule, the statistician shifted the sequence.
6. Codes were kept in separate envelopes for each day for each child.

7. For one child, the parents shared observations (one morning, one afternoon). Both completed total day ratings.
8. Conners Parent Symptom Questionnaire; Children's Behavior Inventory; Missouri Checklist; Oregon Research Institute Checklist; Werry-Weiss-Peters Activity Scale; Classroom Behavior Inventory. Total was 341 items. The same approach was used with the ten children in preschool or elementary school, but teacher ratings proved undependable.
9. Correlations among global measures (counts per hour, Conners Scale, and overall day rating) tended to be substantial, usually with values about $r = .70$.
10. Randomization (or permutation) tests are distribution-free. They were introduced by Fisher [R. A. Fisher, *The Design of Experiments* (Oliver & Boyd, London, ed. 5, 1949), p. 44]. We thank K. R. Gabriel, University of Rochester, for guiding us to the relevant literature and for discussions. As noted by Gabriel, "Their basic concept is well understood: It is that of comparing the experimental outcome with a sample of re-randomizations of the same experimental material." The computer programs came from E. S. Edgington, *Randomization Tests* (Dekker, New York, in press). We used them to perform *t*-tests. Since data such as the current experiment provides can be permuted in millions of ways, the computer program randomly selects 10,000 permutations for the analysis.
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Neurotransmitter Release from a Vertebrate Neuromuscular Synapse Affected by a Food Dye

Abstract. *The food dye erythrosine (Erythrosin B; FD & C No. 3) was applied to isolated neuromuscular synapses in the frog, and its effects on the spontaneous quantal release of acetylcholine were examined with electrophysiological techniques. At concentrations of 10 μM or greater this anionic dye produced an irreversible, dose-dependent increase in neurotransmitter release. This increase did not depend on the presence of calcium ions in the bathing medium. These results suggest that erythrosine might prove a useful pharmacological tool for studying the process of transmitter release, but that its use as a food additive should be reexamined.*

Some food additives have been reported to cause behavioral changes in laboratory animals and humans (*1*), but the validity of these claims has been widely discussed (*2*). By determining whether additives modify some function of individual neurons or interneuronal communication, one might gain a better appreciation for their potential to modify more complex neurological systems. Studies on single invertebrate neurons have revealed that the xanthene dyes used in foods, drugs, and cosmetics alter the permeability of neuronal membranes (*3*). Since changes in the permeability of nerve terminals play an important role in neurotransmitter release, and since many substances that alter neuronal membrane permeability are often more potent in altering synaptic transmission (*4*), we were interested in determining whether food dyes were also active at synaptic junctions. We have, therefore, examined the effects of the widely used food dye erythrosine (Erythrosin B; FD & C Red No. 3) on frog neuromuscular junctions. We report here that erythrosine irreversibly increased transmitter release when applied to the frog's synapse. Experiments directed at elucidating the basis for this effect indicate that the dye-induced transmitter release cannot be readily attributed to an increase in ionized calcium within the presynaptic nerve terminal and suggest that erythrosine alters the release process independently of calcium.

Experiments were performed on the isolated cutaneous pectoris nerve-muscle junction (*5*) of *Rana pipiens*. Spontaneous miniature end-plate potentials (MEPP's), an indicator of transmitter release from the presynaptic nerve terminal (*6*), were recorded from muscle fibers through the use of conventional intracellular recording techniques. Addition of erythrosine (*7*) to the normal frog Ringer bathing medium (*8*) at concentrations as low as 10 μM produced an increase in MEPP frequency (Fig. 1). The MEPP frequency increased in an approximately exponential manner after application of the dye, as revealed by the linear relationship between the logarithm of MEPP frequency and time (Fig. 2). The rate of increase depended on the dye concentration applied. A tenfold increase in frequency was attained within 20 minutes of adding 100 μM erythrosine but occurred about 40 minutes after adding 20 μM dye. Prolonged washing of a dye-treated preparation halted the exponential rise in the frequency of MEPP's, but did not decrease the frequency toward control levels even after more than 2 hours of continuous rinsing with dye-free Ringer (Fig. 2). Prolonged exposure to high concentrations of dye often caused a subsequent decline in the frequency of MEPP's, a result observed after approximately 60 minutes of exposure to 100 μM dye but not after 2 hours in 20 μM dye. Since high dye concentrations produce very high rates of release, depletion of transmitter stores within the nerve terminal (*9*) is a possible cause of this decline.

The mechanism underlying the increase in transmitter release produced by erythrosine is unclear. The frequency of MEPP's is thought to depend on the concentration of calcium within the presynaptic nerve terminal (*5, 10*). To determine whether dye treatment produced an influx of calcium into the nerve terminal, preparations were bathed in calcium-free Ringer to which the calcium-chelating agent, the sodium salt of EGTA (1 mM), was added (*11*). Addition of erythrosine in calcium-free Ringer still produced an increase in MEPP frequency (data not shown), indicating that dye-induced release does not depend on the presence of calcium in the external medium and is therefore not due to calcium entry. Further, other experiments suggest that erythrosine is not triggering release by freeing calcium sequestered intracellularly (*12*). Thus the dye's action does not appear to depend on calcium.

Examination of the effects of erythrosine and related dyes on the properties of other cells might suggest a mode of action. Erythrosine and its analogs alter the membrane permeability of molluscan neurons (*3*) and sea urchin eggs (*13*), thereby altering the membrane potential of these cells. Dye-induced changes in the membrane potential of the presynaptic terminal would most likely in-

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