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.
11. Only in one subject did infractions occur more than sporadically.
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16. This project was supported by contract 223-76-2040 with the Food and Drug Administration, by funds from the Kaiser Foundation Research Institute, by grant MH-11752 from NIMH, by grant ES-01247 from NIEHS, and by a contract with the U.S. Department of Energy at the University of Rochester, Department of Radiation Biology and Biophysics, and has been assigned Report No. UR 3490-1670. We thank T. Sobotka of FDA for intellectual support; G. Lee and I. Childers of KFRI for administrative support; E. Scott and H. Davis for statistical advice; M. Turner for designing the cartoon labels for the drink bottles; R. von Ehrenbrook, S. Garay, O. Gishinsky, T. Stein, and M. Yurko for time and energy; and the 22 families for participating in the experiment.
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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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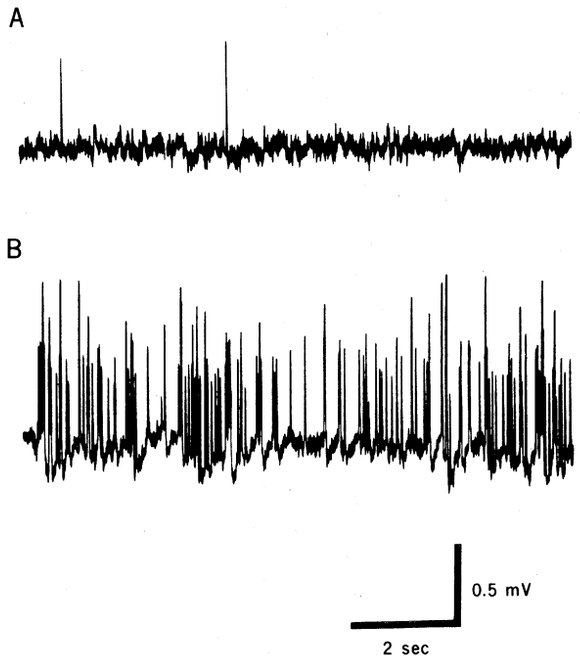

Fig. 1. Intracellular recording (a-c coupled) from an isolated frog neuromuscular junction. MEPP's occur infrequently (0.1 to 0.5 per second) in normal frog Ringer solution (A), but their frequency is increased markedly within minutes (in this case 40 minutes) after 100 μM erythrosine is added to the medium (B). Comparable increases in release are observed approximately 100 minutes after treatment with 10 μM dye solutions.

fluence neurotransmitter release through an action on the voltage-sensitive calcium permeability of the terminal. Since our experiments reveal that the presence of calcium in the extracellular medium is not necessary for dye-induced release, we do not believe that a change in terminal membrane potential, and hence permeability, is the basis for the dye's action. A photodynamic action based on the ability of dyes to absorb light and convert it to energy that modifies nerve membranes has been proposed for the effect of other xanthene dyes on membrane permeability of the squid axon (*14*). Experiments performed at different light intensities reveal no dependence of erythrosine-induced transmitter release on ambient light levels, which indicates that a photodynamic action is not responsible for the effects we have observed. Whatever the dye's mechanism, its ability to alter the release of neurotransmitter independently of calcium, when combined with its well-defined physicochemical properties and the availability of a variety of structural analogs, suggest that it may complement the currently available presynaptic neurotoxins as a pharmacological tool in the study of neurotransmitter release mechanisms (*15*).

Our observation that a widely used food coloring agent, such as erythrosine (*16*), dramatically and irreversibly alters synaptic transmission at low doses is consistent with previous studies suggesting that this and other food additives can alter behavior. While it may be tempting to use these in vitro findings to support claims that these substances would cause behavioral changes when ingested by laboratory animals or humans, such conclusions are premature until it can be determined whether this and other additives have access to the central nervous system. It is not yet known how much of the ingested dye [its estimated maximum daily ingestion was 2 mg per person in 1968 (*16*)] is free in the blood or how readily it crosses the blood-brain barrier.

In sum, the actions of erythrosine on vertebrate neuromuscular transmission indicate that this food additive may alter the function of more complex systems and, thus, that its continued use as an additive should be reconsidered. The ability of this substance to alter release of neurotransmitter independently of calcium suggests that it may be useful as a pharmacological tool in further elucidating the process of transmitter release (*17*).

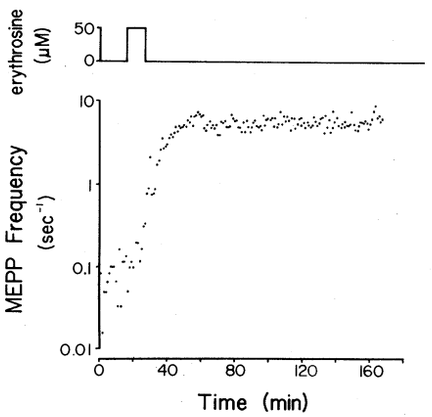

Fig. 2. Kinetics and irreversibility of dye-induced changes in transmitter release. MEPP frequency is increased by exposing the neuromuscular junction to 50 μM erythrosine for 15 minutes. Continuous rinsing with dye-free normal Ringer at a rate of approximately 10 ml/min gradually halted the exponential rise in frequency but gave no indication of reversing it, even after more than 2 hours.

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17. *Note added in proof:* Since we submitted this manuscript, two reports have appeared which demonstrate that erythrosine also inhibits neurotransmitter uptake [J. A. Lafferman and E. K. Silbergeld, *Science* **205**, 410 (1979); W. J. Logan and J. M. Swanson, *ibid.* **206**, 363 (1979)].
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