Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2914–2918, July 1977
Cell Biology

Food, drug, and cosmetic dyes: Biological effects related to lipid solubility

(molluscan neurons/toxicity/uncouplers/anticoagulants/brain uptake)

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Communicated by Harry Grundfest, April 29, 1977

ABSTRACT **Food, drug, and cosmetic dyes of the xanthane type (analogs of fluorescein) were applied to isolated molluscan ganglia and changes in the electrophysiological properties of identified neurons were monitored. The synthetic coloring agents increased the resting membrane potential and conductance of the neurons in a dose-dependent manner by increasing the potassium permeability of the membrane relative to that of other ions. The relative activity of these anionic dyes was highly correlated with their lipid solubility. The structure–activity study of the effects of the dyes on molluscan neurophysiology provides a basis for estimating the toxicity and brain uptake of the dyes in vertebrates, and predicting their effects on metabolism and blood clotting.**

Synthetic dyes have been a constituent of foods, drugs, and cosmetics for more than 100 years, and the safety of such additives has been of concern for almost as long (1). The intended function of these additives has been to enhance the physical appearance of a product, and some proof of their safety, which has in the past been either assumed or ignored, is now required in many countries before they can be incorporated into items intended for human consumption (1–5). Despite such precautions several coloring agents were used for decades before they were ruled unsafe, and the use of other dyes is currently under review because deleterious effects have been discovered or are suspected (1, 5). Interest in the biological activity of artificial colorants has recently been renewed following the claims that a red food dye (FD&C Red No. 2) is carcinogenic (6) and that some food additives, and colorants in particular, may be responsible for producing behavioral changes and learning disabilities in children (e.g., hyperkinesis, minimal brain dysfunction) (7–9).

The present study was undertaken in order to determine (*i*) whether some types of synthetic dyes, which are added to foods and drugs purely for cosmetic purposes, are in fact biologically active compounds, and (*ii*) the physicochemical properties of the compounds responsible for their activity. It was found that xanthane type dyes alter the physiological characteristics of invertebrate neurons and that their biological activity is highly correlated with the compounds' lipid solubility. The structure–activity study of the dyes' effects on neurons provided a basis for estimating their toxicity and brain uptake in vertebrates, and predicting their effects on metabolism and blood clotting.

METHODS AND MATERIALS

Experiments were performed primarily on the buccal ganglia of the marine mollusc *Navanax inermis* (obtained from Pacific Biomarine Supply Co., Venice, CA). This invertebrate nervous system was used previously to provide insights into the cellular mechanism of action of nonnarcotic analgesics in vertebrates (10, 11) and it was hoped it might serve a similar function for the current problem. The ganglion was isolated from the animal, pinned to Sylgard (Dow Corning, Midland, MI) in the bottom of a small plastic dish (5 ml) and usually bathed in a low-potassium physiological saline consisting of 500 mM NaCl, 1 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM Tris·HCl at pH 7.8. The potassium concentration was increased by isotonic substitution for sodium. The capsule enveloping the ganglion was cut, exposing large, previously identified neurons (12). These neurons were impaled with double-barreled micropipettes with tip diameters of 2 μm or less, and filled with 3 M KCl. The neuronal membrane potential was monitored with one barrel using standard electrophysiological technique (12). The second barrel was connected to a source of constant current pulses so that total membrane conductance could be monitored.

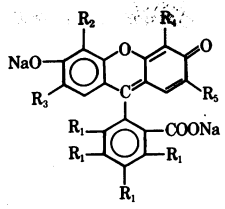
The compounds selected for testing belong to the xanthene group of dyes and are derivatives of fluorescein (Table 1). Fluorescein itself is frequently used in angiography to visualize the blood vessels of the eye (17) and some of its derivatives are used to color foods and drugs (4). The dye to be tested in the current system was dissolved in the physiological saline just prior to use, and was applied to the impaled neurons by exchanging the normal physiological medium for one containing the dye.

RESULTS

The application of the fluorescein dyes to the molluscan ganglia produced a rapid increase in the membrane potential and conductance of the impaled neuron (Fig. 1). The amplitude of the response to a particular dye was variable and dependent upon several factors, including the identity of the neuron impaled, the animal, the time of year the experiment was performed, and the number of previous exposures to that dye or others. Because of desensitization in the response to repeated dye applications and/or a slow irreversible decline in the membrane potential produced by some of the dyes, complete dose–response curves were rarely obtained. In order to have a common base with which to compare the relative effectiveness of the several dyes, the response of a neuron to each dye was compared with its response to a preceding and subsequent application of 12 mM salicylate, an organic anion of related structure known to produce similar effects in this system (18–21). This concentration of salicylate produces half the maximum change in membrane potential and conductance and its effects are reversible. The concentration of a particular dye that produced an increase in membrane potential equal to that produced by 12 mM salicylate was considered the concentration that would produce half the maximum change in membrane

Abbreviations: FD&C denotes approval by the United States Food and Drug Administration for use in food, drugs, and cosmetics; D&C, approved for drugs and cosmetics.

Table 1. Biological activity and partition coefficient of fluorescein analogs



Compound[a]	Colour Index (1971) no.[b]	R_1	R_2	R_3	R_4	R_5	log P[c]	C_{50}, mM Observed[e]	C_{50}, mM Predicted[f]	Predicted values I_{50},[g] mM	Predicted values F,[h] mM	Predicted values LD_{50},[i] mmol/kg	Predicted values BUI,[j] %
1. Fluorescein (D&C Yellow No. 8)	45350	H	H	H	H	H	−4.77	200	300	50*	3200*	>10*	—*
2. D&C Orange No. 9	45365	H	Cl	H	Cl	H	−3.77	—	38	11*	1000*	>10*	2
3. Eosin 2J (D&C Orange No. 6)	45370	H	Br	H	Br	H	−3.02	—	22	4	400*	>10*	4
4. Erythrosin Y (D&C Orange No. 11)	45425	H	I	H	I	H	−2.46	—	10	2	200*	>10*	10
5. Eosin B	45400	H	Br	NO_2	Br	NO_2	−2.18[d]	8	6	1	135*	>10*	14
6. D&C Red No. 25	45366	H	Cl	Cl	Cl	Cl	−1.97	—	5	0.9	105*	>10*	19
7. Eosin Y (D&C Red No. 23)	45380	H	Br	Br	Br	Br	−1.27	4	2	0.3	47	>10*	40
8. Erythrosin B (FD&C Red No. 3)	45430	H	I	I	I	I	−0.15[d]	0.2	0.3	0.06	10	8	82
9. Ext. D&C Red No. 5	45435	di-Cl	I	I	I	I	1.44	—	0.03	0.006	2	2	86*
10. Phloxine B (D&C Red No. 28)	45410	Cl	Br	Br	Br	Br	2.02	0.01	0.01	0.003*	1	1	65*
11. Rose Bengal	45440	Cl	I	I	I	I	3.14	—	0.002	0.0005*	0.2*	0.6	25*

[a] FD&C denotes approval by the United States Food and Drug Administration for use in foods, drugs, and cosmetics; D&C, drugs and cosmetics; Ext., external. Fluorescein, Eosin B, Eosin Y, Phloxine B, and Rose Bengal are certified dyes obtained from Matheson, Coleman, and Bell, Norwood, OH; Erythrosin B was a gift from M. Chin, Stange, Co., Chicago IL.

[b] The Colour Index number provides unique identification of the compounds (4).

[c] With the exception of compounds 5 and 8, the octanol/water partition coefficients, P, of the ionized form of the molecules were calculated by applying the additive-constitutive rules established by Hansch *et al.* (13).

[d] Octanol/water partition coefficients of ionized form of these molecules determined experimentally by Bio- and Pharmaceutical Analysis Section, Midwest Research Institute, Kansas City, MO. Sodium was the co-ion in all cases. The pK_a values of Eosin and Erythrosin B were found to be 4.95 and 5.04, respectively.

[e] C_{50} = concentration, estimated from experimental observations, that would produce half the maximal increase in membrane potential of identified molluscan neurons.

[f] C_{50} predicted from least squares fit of data points (Eq. 1).

[g] I_{50} = concentration that should inhibit oxidative phosphorylation of isolated rat liver mitochondria by 50%, predicted from an equation describing similar activity of salicylate analogs (see *text*).

[h] F = concentration that should dissolve a standard clot of human plasma in 24 hr, predicted from an equation describing similar activity of salicylate analogs (14).

[i] LD_{50} = concentration that should kill 50% of the mice that receive an intravenous administration, predicted from an equation describing similar activity of benzoate analogs (15).

[j] BUI = Brain uptake index or ratio of concentration of organic anion to tritiated water which should be found in rat brain to that injected into the internal carotid artery 15 sec earlier (16).

* The partition coefficient for these compounds lies outside the range of data from which the equation was originally calculated.

potential, C_{50}, if complete dose–response curves could be obtained. The values of C_{50} ranged from 10 μM for Phloxine B (D&C Red No. 28) to about 200 mM for Fluorescein (D&C Yellow No. 8), and the membrane potential change produced by applying a concentration equal to C_{50} varied from 2 to 20 mV. The C_{50} for Erythrosin B (known as Colour Index Food Red 14 in Europe and FD&C Red No. 3 in the United States), the only analog approved for use as a food coloring, was about 200 μM. Although the immediate effects of the dyes on membrane potential and conductance were reversible by washing with dye-free medium, exposure to several of the dyes (Eosin Y, Erythrosin B, Phloxine B, and Rose Bengal) also initiated a slow irreversible decline in the resting membrane potential. In all cases the connective tissue surrounding the ganglion and other tissue inside the ganglion remained stained by the dye even after extensive washing with saline. This observation is consistent with the fact that some of the compounds are used extensively as biological stains for diagnostic and histological purposes, and have various degrees of affinity for proteins and cellulose (22).

To determine the ionic basis for the increase in membrane potential and conductance caused by the dyes, the dependence of the membrane potential on the concentration of potassium in the external medium was examined in the presence and absence of the dyes. The results of one such experiment are illustrated in Fig. 2. Erythrosin B (0.4 mM) caused an increase in the variations of the membrane potential with changes in the external potassium concentration (Fig. 2*A*), and also caused a 50% reduction in the effective membrane resistance (Fig. 2*B*). The decrease in resistance was not due to the change in membrane potential because the membrane does not show rectifying properties over this range of potentials (19). A similar increase in the dependence of membrane potential on external potassium would be observed if the dyes reduced the contribution that ions other than potassium made to the membrane potential (18, 20). In either case one may conclude that the increase in

FIG. 1. Erythrosin B (0.5 mM), indicated by bar, causes a reversible increase in the membrane potential of identified neuron M-R, from −58 to −65 mV, and a 20% increase in membrane conductance, as revealed by the decrease in the membrane potential change produced by three, brief constant current pulses. Medium contained three times the normal magnesium concentration in order to minimize synaptic activity.

membrane potential and conductance was apparently due to an increase in the conductance of the membrane to potassium relative to other ions.

A quantitative analysis of the relationship between the physicochemical characteristics of the dyes and their ability to alter membrane potential revealed that the dyes' biological activity was highly correlated with their octanol/water partition coefficient (a measure of lipid solubility). The logarithm of the relative activity, given by the inverse of the molar concentration required to produce half the maximum change in membrane potential (C_{50}), was linearly related to the logarithm of the octanol/water partition coefficient, P, of the ionized form of

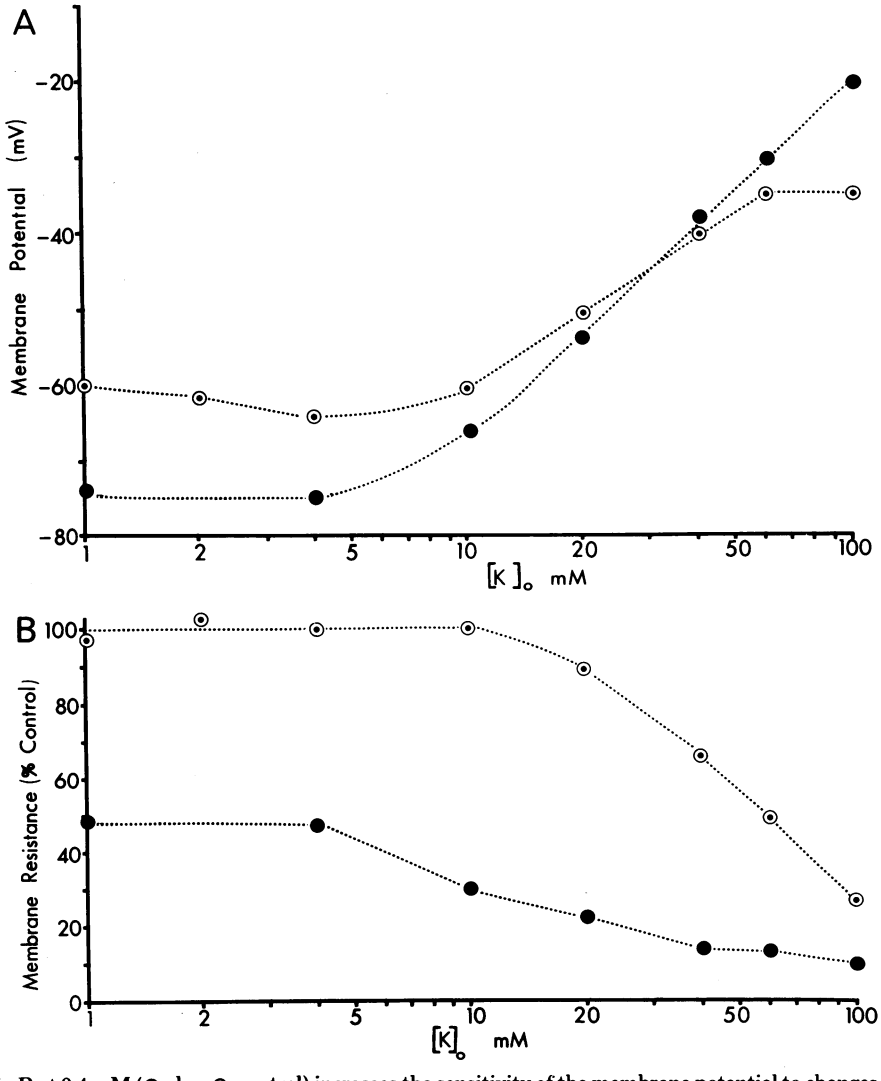


FIG. 2. (*A*) Erythrosin B at 0.4 mM (●, dye; ⊙, control) increases the sensitivity of the membrane potential to changes in the external potassium concentration, $[K]_o$, and (*B*) decreases the membrane resistance at any $[K]_o$. The potassium concentration was increased by isotonic substitution for sodium in the medium. Upon changing $[K]_o$ the membrane potential reaches a new level within about 2 min of superfusing the ganglion at 50 ml/min with more than 10 times the volume of the chamber. Thereafter, the potential remains steady. Example from identified neuron M-LD.

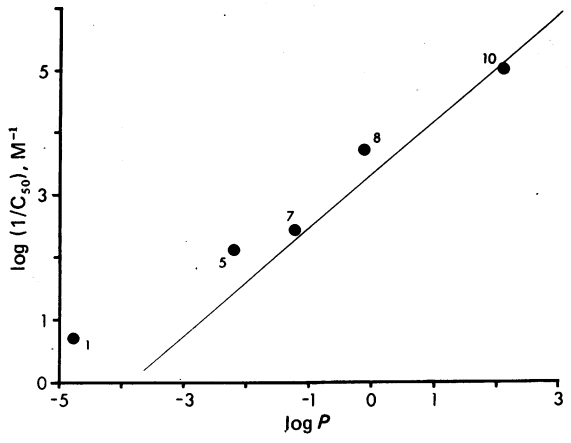


FIG. 3. The concentration of fluorescein dyes that produces half the maximum increase in neuronal membrane potential, C_{50}, is a function of the octanol/water partition coefficient of the ionized form of the dye, P. C_{50} values of the dyes were estimated by determining the dye concentration that produced the same change in membrane potential as 12 mM salicylate. The least squares regression line [$\log (1/C_{50}) = 0.87 \log P + 3.32$] of the activity of a variety of other organic anions in this system as a function of partition coefficient (21) is superimposed on the data points for the fluorescein dyes. Numbers refer to compounds listed in Table 1.

the molecule (Fig. 3). A linear regression line for these data is presented in Eq. 1. The numbers in parentheses are 95% confidence intervals.

$$\log (1/C_{50}) = 0.65(\pm 0.18) \log P + 3.60(\pm 0.46). \quad [1]$$

A statistical analysis of these data ($n = 5$) revealed a correlation coefficient, $r = 0.989$ ($r^2 = 0.978$), and a standard error of the estimate, $s = 0.279$. The F ratio from an analysis of variance was $F_{1,3} = 115.2$, which is greater than the F ratio at the confidence level (α) of 0.005, $F_{1,3} = 55.6$. Although the partition coefficient of the undissociated dyes is probably about 10^4 times greater than that of the ionized form (23, 24), the latter has been used here because (*i*) at a pH of 7.8 about 99.9% of any one dye is in the ionized form ($pK_a \simeq 5$), and (*ii*) the ionized form is likely to be about 100 times more active than the undissociated form (21, 24).

In all respects the response of the neurons to these anionic coloring agents resembled their response to salicylates (18–21), and the relation between the activity and the partition coefficient of the dyes was comparable to that previously described for a variety of other organic anions of very different structure (25) (Fig. 3). The results therefore confirm and extend the concept that the ability of organic anions to alter the membrane physiology of these invertebrate neurons can be predicted quite accurately from their partition coefficient (19, 21, 25). On this basis I have calculated the concentration of other coloring agents, not tested here, which would produce half the maximum change in membrane potential and conductance (Table 1).

The systemic toxicity (15, 16) and brain uptake (16) of carboxylic acids in vertebrates, and the effects of these anions on vertebrate metabolism (26) and blood coagulation (14) is also highly correlated with their partition coefficient. One would anticipate, therefore, that the agents used to color foods, drugs, and cosmetics would also alter these processes to an extent dependent upon their respective lipid solubilities. In fact there is evidence that some structurally related anionic dyes, such as sulfonephthalein derivatives, uncouple rat liver mitochondria (27) or otherwise alter the properties of rat mitochondrial membranes (28) roughly in relation to their lipid solubility, and that a variety of other anionic dyes have anticoagulant properties (29). Other organic anions have been shown to cross the blood–brain barrier (30) in a manner highly correlated with their partition coefficient (16, 17). Moreover, the neurotoxicity of organic anions has been shown to be highly correlated with the drug's partition coefficient (15–17), and this may be in part a reflection of their ability of penetrate the blood–brain barrier. The anionic dyes considered in the present report may also penetrate the blood–brain barrier in the same manner as other, noncolored organic anions.

Assuming that the xanthene dyes act like a variety of other organic anions, I have calculated for each of the dyes listed in Table 1, (*i*) the molar concentration, I_{50}, that should inhibit by 50% the oxidative phosphorylation of isolated rat liver mitochondria, using Eq. 2, (H. Levitan and J. L. Barker, unpublished work),

$$\log (1/I_{50}) = 0.64(\pm 0.12) \log P + 4.3(\pm 0.19), \quad [2]$$

(*ii*) the molar concentration, F, that should completely break up human blood clots *in vitro* within 24 hr, using Eq. 3 (14),

$$\log (1/F) = 0.52(\pm 0.10) \log P + 1.98(\pm 0.14), \quad [3]$$

and (*iii*) the concentration, LD_{50}, in mmol/kg, that should kill 50% of a population of mice within 24 hr after they receive an intravenous injection, using Eq. 4 (15, 16),

$$\log (1/LD_{50}) = -0.06(\pm 0.06) (\log P)^2 + 0.51(\pm 0.20) \log P - 0.82(\pm 0.16). \quad [4]$$

(*iv*) In a similar manner I have estimated the brain uptake index, BUI, or the ratio of the concentration of dye relative to tritiated water (expressed in %) that should be found in the brain of rats 15 sec after intracarotid injection, using Eq. 5 (16),

$$\log \text{BUI} = -0.10(\pm 0.07) (\log P)^2 + 0.14(\pm 0.16) \log P + 1.94(\pm 0.20). \quad [5]$$

Previous studies on the metabolic (27, 28), toxic (31), and anticoagulant (29) effects of a variety of anionic dyes can now be placed in this more general context.

DISCUSSION

The present study shows that a group of anionic dyes which are fluorescein analogs alter the membrane physiology of molluscan neurons. The relative activity of the dyes is highly correlated with their octanol/water partition coefficient, increasing in potency with increasing lipid solubility. The mechanism by which these anionic dyes modify the relative membrane permeability may be similar to that proposed for the action of other organic anions (20, 21). In that case it was suggested that the anions adsorb to and thereby modify the membrane's anionic field strength. A change in membrane surface charge is also possible, however (32).

Because the effects produced by these anionic dyes are identical in many respects to those produced by a large variety of other organic ions, the suggestion has been made that the fluorescein analogs should be active in other systems where the biological activity of organic anions has been found to be highly correlated with the molecule's partition coefficient. On this basis the toxicity and brain uptake of the dyes in vertebrates has been estimated, and their inhibition of oxidative phosphorylation and blood clotting has been predicted. It is hoped that the work will also provide a basis for anticipating other biological effects of artificial coloring agents, and a rationale for selecting relatively

inocuous dyes and establishing safe levels for their internal consumption and external use.

In support of the nonspecific biological activity of the fluorescein dyes predicted in the current paper, it has recently been found that these dyes also reversibly inhibit fertilization in echinoderms with a relative potency correlated with their lipid solubility (33), and reversibly modify transmission at the frog neuromuscular junction (34).

I thank Drs. E. J. Carroll, E. J. Elliott, H. Gainer, K. B. Levitan, and S. I. Rapoport for critically reading the manuscript. This research was supported by a grant from the National Science Foundation (GB-43141).

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