possibility that neurotransmitters or neurohormones released from the abdominal viscera may have circulated to the bronchial muscle and contributed to the maintenance of bronchodilation. Accordingly, we made an incision through the abdomen and diaphragm in three cats and sectioned the right and left vagus nerves 2 cm proximal to the esophageal hiatus. These animals continued to respond to stimulation of the cervical vagus nerves with prolonged bronchodilation. Thus it would appear that the common mechanisms for rapidly terminating neurotransmitter action, such as enzymatic degradation and neuronal uptake, are poorly developed or inoperative in the nonadrenergic system of airway smooth muscle control. It is also possible that this system operates through a mechanism fundamentally different from the usual forms of synaptic transmission; for example, a neuroparacrine control system in which chemical mediators are released from neurosecretory fibers and slowly diffuse through the extracellular space to reach their targets (*10*).

The physiological significance of the nonadrenergic inhibitory innervation of the airways is unknown. Possibly it is the remnant of a primitive inhibitory nervous system. Alternatively, it may serve as the dominant inhibitory nervous system in certain mammalian airways or provide redundancy for the inhibitory actions of adrenergic nerves and circulating catecholamines. Richardson and Beland (*6*) found no evidence of adrenergic inhibitory fibers in human bronchial muscle with pharmacological or histochemical techniques, and speculated that the nonadrenergic inhibitory system might function as the principal inhibitory nervous system for smooth muscle in human airways. The tracheobronchial tree and the gastrointestinal tract are both endodermic in origin, and in the latter a nonadrenergic inhibitory system is known to be the most important mechanism controlling the relaxation phase of peristalsis and the function of many sphincters (*11*). When the system is absent, as in humans with Hirschsprung's disease (*12*) or in animals whose intramural ganglion cells have been anoxically destroyed (*13*), the result is loss of inhibitory control and spasm of the gastrointestinal smooth muscle. If the nonadrenergic inhibitory system in airway smooth muscle plays a correspondingly important role in regulating bronchial muscle tone, then, as suggested by Richardson and Bouchard (*4*), a defect in the system could account for the hyperreactive airways of victims of asthma and chronic bronchitis. Such a defect could also account for the common but enigmatic clinical observation that beta adrenergic blocking agents cause bronchoconstriction in asthmatic patients but not in nonasthmatics.

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Inhibition of Cell Division and Growth by a Redox Series of Cyanine Dyes

Abstract. *A series of cyanine dyes used in photography, with reduction potentials from −1.35 to −0.20 volts, were tested for their ability to inhibit mitosis and cell growth in fertilized sea urchin eggs. Low concentrations of dyes with reduction potentials more negative than −1.0 volt generally inhibited mitosis and growth, whereas those with more positive reduction potentials did not. The active dyes penetrated the cell, entered all subcellular compartments, were bound to numerous macromolecules, and inhibited synthesis of macromolecules. Thus mitosis and growth may be retarded with substances that can alter electrochemical activity in cells.*

Electrochemical studies of dyes used in photography to control silver halide responses in emulsions (*1*) have provided a dye series with a wide range of reduction and oxidation potentials. Using this dye series, Gilman (*2*) determined the electrochemical potentials that will shut off the photoelectronic activity of silver halide substrates. The effective control of electronic events achieved with this series led us to the idea that these same dyes might also be used to modify electronic events in living cells. Control of electrochemical potentials in biochemical reactions could provide new ways of limiting abnormal or infectious cell growth in disease.

The electrochemical properties of these cyanine dyes were measured polarographically by the method of Large (*3*). A more negative reduction potential (E_R) indicates greater difficulty in reducing a dye; a more positive oxidation potential indicates greater difficulty in oxidizing the dye. When used on photographic silver halide emulsions, dyes with E_R values more negative than −1.0 V are the most efficient spectral sensitizers, and dyes with less negative E_R values are desensitizers of the normal photographic process (*2*).

Certain cyanine dyes are useful for treating parasitic infections (*4*) and can inhibit respiratory chain reactions involving electron transport (*5*). Still, few attempts have been made to systematically relate the electrochemical properties of cyanine dyes to their biological actions. The study described here was performed to determine whether such a correlation applies to fertilized sea urchin eggs.

A strong relation was found between the E_R of a cyanine dye and its ability to inhibit cell division. Methanolic solutions of 22 dyes at concentrations of 10^{-6} to $10^{-5}M$ were added to suspensions of fertilized eggs of the sea urchins *Arbacia punctulata* and *Lytechinus variegatus* (200,000 eggs per milliliter) 3 minutes after fertilization. A light microscope was used to observe and photograph the ability of the eggs to divide and form the mitotic apparatus.

The dyes were assigned a number from 0 to 5+, with 5+ representing complete mitotic inhibition and 0 indicating none. With few exceptions, the more negative the E_R value of the dye, the greater the inhibition of mitosis (Fig. 1). Dyes 15 and 20 showed anomalous behavior photographically and in our biological model. The inability of dye 5 ($E_R = -1.12$ V) to inhibit mitosis may be

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related to its poor solubility in seawater. Although properties other than E_R may play a role in the net biological effects of the dyes, there is, as in photography, a remarkable correlation between a dye's biological action and its E_R.

We attempted to compare the biological action of several dyes that vary only slightly in their structures and molecular weights but widely in their E_R values. As shown in Fig. 1, the benzoxazole dyes 2, 3, and 4 (E_R values of -1.26, -1.31, and -1.28 V, respectively) strongly inhibit mitosis, but the cyano-substituted benzoxazole dye 9 (E_R, -0.99 V) is totally inactive. In the benzothiazole series, dye 8 (E_R, -1.00 V) is an active inhibitor, but the nitro-substituted dye 17 (E_R, -0.58) is inactive.

Figure 2 shows control (methanol-treated) eggs and dye-treated eggs 30 and 60 minutes after fertilization. Although dyes with E_R values more negative than -1.00 V did not prevent the formation of the mitotic apparatus after 30 minutes, no cell division occurred after 60 minutes (as did occur in the control eggs or in eggs treated with dyes with less negative E_R values). Eggs exposed to dyes with highly negative E_R values exhibited enhanced color compared to the controls or to eggs exposed to dyes with less negative E_R values.

Using fluorescence microscopy, we found that all parts of eggs treated with dye 2 were strongly fluorescent, whereas those treated with dye 9 were fluorescent at background levels only. The fluorescence of both dyes in solution is similar quantitatively and qualitatively, as determined with a double-beam spectrofluorometer (Perkin-Elmer model 512). Fluorescence techniques were also used to estimate the ability of these dyes to penetrate the egg membranes, reach various subcellular compartments, and bind to macromolecules.

Fluorescence emissions of bound dyes were at 5 to 10 mm longer wavelengths than those of the free dyes. Eggs treated with the dyes for various periods were thoroughly washed and homogenized in 0.01*M* tris-EDTA (*p*H 7.3), and the homogenate was separated centrifugally into the nuclear (800*g*), mitochondrial (10,000*g*), microsomal (100,000*g*), and supernatant fractions. The macromolecules in the supernatant fractions were precipitated with trichloroacetic acid (TCA) or ethanol and analyzed, after dissolution in sodium dodecyl sulfate (SDS), by fluorescence spectral measurements.

About 20 percent of each dye with an E_R value more negative than -1.0 V penetrated the eggs, and the dyes were firmly bound to macromolecules in all subcellular compartments. The dyes could not be removed from the macromolecules of the subcellular fractions by dissolution in and dialysis against 0.1 percent SDS for 24 hours. The dyes with E_R

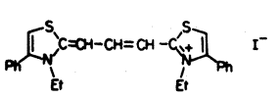
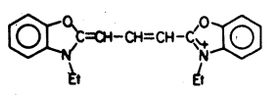
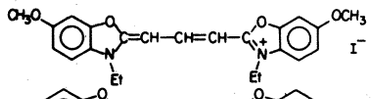
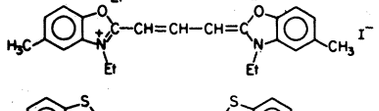
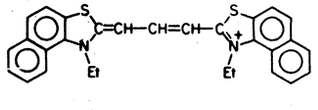
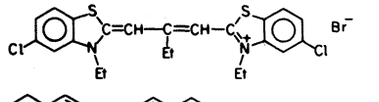
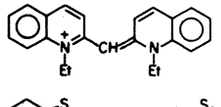
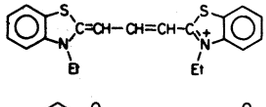
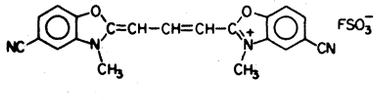
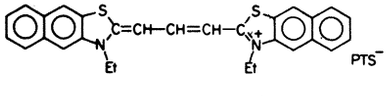
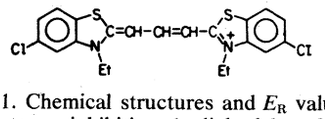
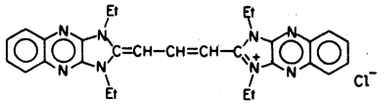
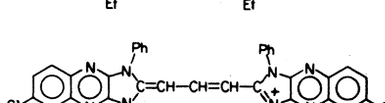
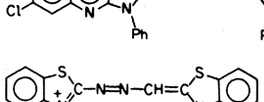
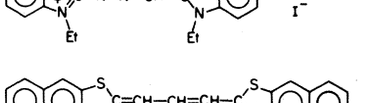
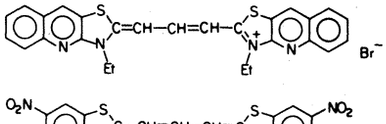
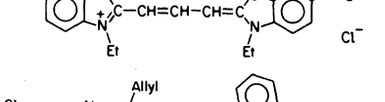
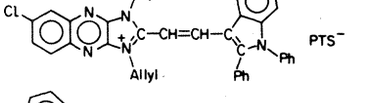
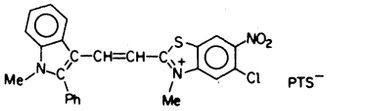
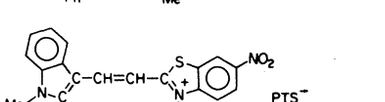
Dye	Structure	E_R (V)	Biological action
1		−1.35	5+
2		−1.26	4+
3		−1.31	4+
4		−1.28	4+
5		−1.12	0
6		−1.06	4+
7		−1.03	5+
8		−1.00	4+
9		−0.99	0
10		−0.90	0
11		−0.86	3+
13		−0.79	0
14		−0.65	0
15		−0.64	2+
16		−0.63	0
17		−0.58	0
18		−0.54	0
19		−0.41	0
20		−0.32	2+
21		−0.20	0

Fig. 1. Chemical structures and E_R values of cyanine dyes and their inhibitory actions on mitosis and DNA synthesis in sea urchin eggs (0 indicates no inhibition; 1, slight delay of division; 2+, 50 percent of eggs divide; 3+, 25 percent of eggs divide; 4+, 10 percent of eggs divide; and 5+, no division).

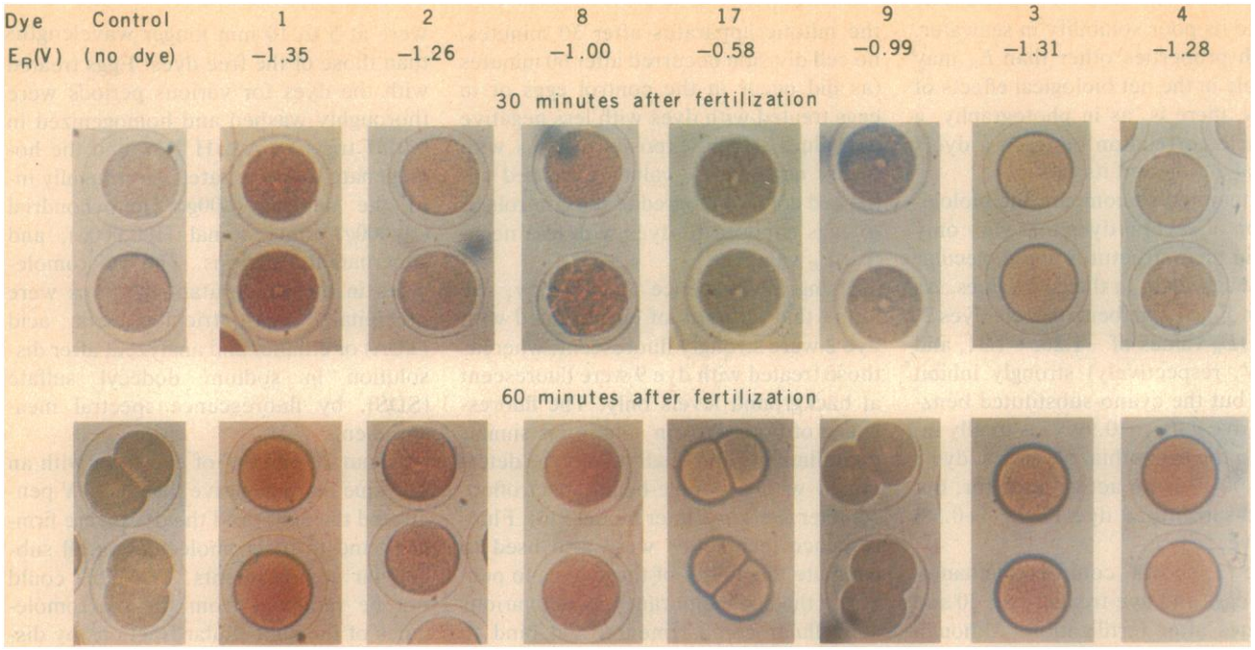


Fig. 2. Color photomicrographs (×200) of sea urchin eggs 30 and 60 minutes after fertilization. Dye was added to seawater containing the eggs 3 minutes after fertilization (final concentration of dye, 25 μg/ml). The dye solvent was methanol (0.025 ml per milliliter of seawater). Mitotic apparatus and fertilization membranes are visible in all eggs after 30 minutes, but division is absent after 60 minutes in eggs treated with dyes having E_R values more negative than −1.0 V.

values less negative than −1.0 V were less firmly bound than dyes with more negative E_R values and did not inhibit mitosis. Dyes that are effective inhibitors of mitosis and of macromolecular synthesis are permanently retained at many biologically active sites in the cells; this is analogous to the photographic process in which the dyes must be adsorbed to silver halide crystals to be active.

Inhibition of cell growth was also assessed by using liquid scintillation counting techniques (*6*) to measure the ability of the eggs to synthesize DNA (to incorporate [^{3}H]thymidine into TCA-precipitated material) and protein (to incorporate ^{14}C-labeled amino acids into TCA precipitates). The relative inhibition of growth by the active dyes was ascertained by serially diluting them in the seawater and determining the lowest dye concentration that would stop mitosis and [^{3}H]thymidine incorporation into DNA [as in (*6*)].

The incorporation of [^{3}H]thymidine into egg DNA was markedly lowered by dyes with E_R values more negative than −1.0 V. Thirty minutes after fertilization, the control eggs had incorporated 110 count/min (above background) per 0.1-ml portion after being washed with 5 percent TCA and dissolved in 0.5*M* NaOH; with dye 9 (inactive) added, 107 count/min was incorporated; with dyes 3 or 4 added, 17 and 18 count/min were incorporated, respectively.

After 60 minutes, the control samples contained 580 count/min; the samples with dye 9, 408 count/min; and the samples with dyes 3 and 4, 19 and 18 count/min, respectively.

These results indicate that there is a strong relation between a dye's E_R and its ability to inhibit macromolecular synthesis. The exceptions may be due to other factors such as dye charge, partition coefficient, stability, and absorption. Dyes that are effective inhibitors of cell division and macromolecular synthesis penetrate the eggs readily and migrate freely to all subcellular compartments, upsetting the natural electrochemical interactions between macromolecules and electrons. A possible site of action of the dyes is the mitochondrial membrane, where active transport of electrons occurs to support cellular respiration and energy supply [for example, production of adenosine triphosphate (ATP)]. A dye with an E_R more negative than that of respiratory chain reactions could occupy electron acceptor sites, turning off the transport of electrons and cellular respiration. A reduction in energy production would slow the synthesis of macromolecules and delay mitosis. Dyes with less negative potentials could block energy production to a lesser degree by accepting or donating electrons further along the chain (*7*). The dyes would thus create electronic barriers to the flow of electrons in key transport systems dependent on their E_R.

Kinnally and Tedeschi (*5*) have already suggested that some cyanine dyes may inhibit respiratory chain activity in electron transport. Szent-Györgyi (*8*) proposed that the control of electrons transferred to many proteins and enzymes may generally affect the efficiency of their action, influencing cell growth potential.

Thus cyanine dyes with highly negative E_R values may be useful as inhibitors of rapid cell growth. Redox potential should be considered when selecting dyes for use in controlling abnormal cell growth and infectious processes in higher animals and man. Hodnett *et al.* (*9*) recently showed a strong correlation between E_R and the antitumor activity of a series of benzoquinone analogs. We speculate that practical means to control cell division and infectious processes can be derived from the experimental and theoretical considerations discussed in this report (*10*).

Note added in proof: Recent experiments have shown a marked lowering of ATP levels in fertilized eggs exposed to the antimitotic dyes with highly negative E_R values.

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11. We thank B. C. Antonellis for her excellent technical assistance.

27 November 1979

Phorbol Ester Action Is Independent of Viral and Cellular *src* Kinase Levels

Abstract. *Treatment of normal chick embryo fibroblasts with phorbol myristate acetate causes those cells to express many of the phenotypic properties of virally transformed cells and also enhances the expression of transformed properties in Rous sarcoma virus–transformed chick embryo fibroblasts. We report here that phorbol myristate acetate has little or no effect on the level of protein kinases encoded by the viral* src *or endogenous* sarc *genes.*

Phorbol esters, the most potent class of tumor promoters, have been shown to induce partial mimicry of the transformed phenotype in normal fibroblasts growing in monolayer culture, to cause enhanced expression in transformed cells of properties frequently associated with the transformed state, and to inhibit terminal differentiation and differentiated cell functions (*1-5*). Because the results of phorbol ester action bear a striking resemblance to the effects on cells induced by the transformation (*src*) gene product of Rous sarcoma virus (RSV), it has been hypothesized that the phorbol esters might act through an effect on the amount or activity of the viral *src* or endogenous *sarc* gene product (*6, 7*). Indeed, Bissell *et al.* (*7*) proposed that the *src* gene product of RSV might better be viewed as a promoter rather than as a carcinogen or "initiator." Recently, substantial progress has been made in identifying, assaying, and characterizing the viral *src* and cellular *sarc* gene product (*8, 9*). The *src* gene of RSV appears to code for a protein of molecular weight 60,000, designated pp60^{src} by Erikson and co-workers (*9*), which can be immunoprecipitated from extracts of RSV-infected chick embryo fibroblasts with antiserum derived from rabbits bearing RSV-induced tumors. The pp60^{src} antigen is capable of transferring ^{32}P-labeled phosphate from γ-^{32}P-labeled adenosine triphosphate (ATP) to the immunoglobulin molecule to which it is bound. Uninfected chick embryo fibroblasts also possess a protein kinase of similar molecular weight which can be immunoprecipitated by the same antiserums (*10, 11*). This protein, the product of the endogenous *sarc* gene, is, however, present at 1 to 2 percent of the level of the viral *src* gene product in RSV-transformed chick embryo fibroblasts.

To determine whether tumor promoters modulate the level of the product

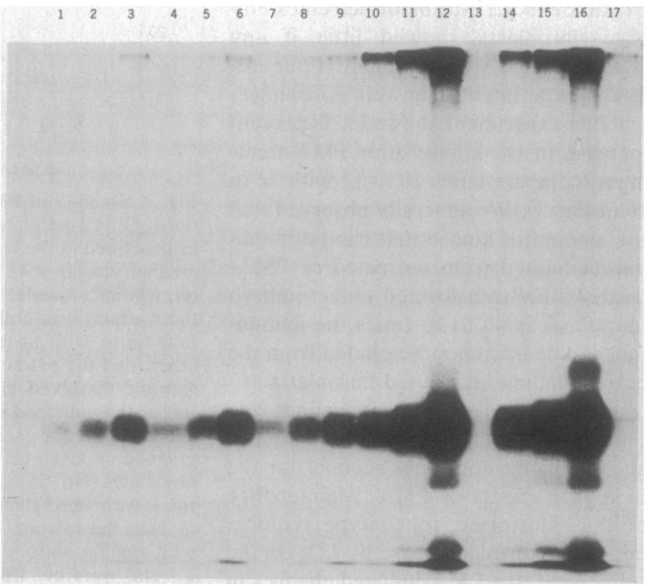

Fig. 1. Effect of PMA on the level of viral *src* kinase or cellular *sarc* kinase. Normal or Schmidt-Ruppin RSV-transformed secondary chick embryo fibroblasts were plated at 4×10^6 or 5×10^6 cells, respectively, per 100-mm dish and grown with daily medium changes in medium 199 supplemented with 10 percent tryptose phosphate broth, 4 percent calf serum, 1 percent heat-inactivated chicken serum, penicillin G (75 U/ml), streptomycin (50 μg/ml), and either dimethyl sulfoxide (DMSO) (0.1 percent, final concentration) or PMA (100 ng/ml, 0.1 percent DMSO, final concentration). Cells were then lysed in buffer containing 1 percent Triton X-100 as described in (*15*). Cell extracts were immunoprecipitated with antiserum from tumor-bearing rabbits (*8*). Antiserums from several rabbits showed broad cross-reactivity with pp60^{src} encoded by several strains of RSV. These serums recognized a protein in uninfected chick embryo fibroblasts that possessed protein kinase activity (*11*). Immune complexes were adsorbed to protein A–Sepharose beads (Pharmacia), washed with Triton buffer, and then assayed for kinase activity. Reaction mixtures were incubated at room temperature for 10 minutes in buffer containing 20 m*M* tris, *p*H 8.1, 5 m*M* $MgCl_2$, 14 m*M* 2-mercaptoethanol, and either 6.6×10^{-8}*M* [γ-^{32}P]ATP (3000 Ci/mmole) when transformed cells were assayed or 2.3×10^{-7}*M* [γ^{32}-P]ATP (3000 Ci/mmole) when uninfected cells were assayed. After incubation, the Sepharose beads were washed and adsorbed proteins were released by boiling in sodium dodecyl sulfate (SDS) and mercaptoethanol and were analyzed by electrophoresis on an 8 percent SDS-polyacrylamide gel. Gels were dried and processed for autoradiography. Tracks 1 to 3 show 100, 200, and 600 μg of protein extracted from quiescent normal chick cells immunoprecipitated with serum from tumor-bearing rabbits (TBR); tracks 4 to 6 show 100, 200, and 600 μg of protein extracted from serum-stimulated normal chick cells immunoprecipitated with TBR serum; tracks 7 to 9 show 100, 200, and 600 μg of protein extracted from normal cells that had been serum-stimulated and treated with PMA; tracks 10 to 12 show 50, 100, and 300 μg of protein extracted from RSV-transformed cells immunoprecipitated with TBR serum; track 13 shows 300 μg of protein extracted from RSV-transformed cells immunoprecipitated with normal rabbit serum; tracks 14 to 16 show 50, 100, and 300 μg of protein from PMA-treated RSV-transformed chick cells immunoprecipitated with TBR serum; track 17 shows 300 μg of protein from PMA-treated RSV-transformed cells immunoprecipitated with normal rabbit serum. In all lanes, ^{32}P-labeled phosphate had been transferred to the 52,000-dalton reduced form of immunoglobulin. For quantitation, this band was cut out from the gel and radioactivity was determined. Under the conditions of the assay, radioactivity was proportional to the amount of active kinase rather than its specific activity (*15*).

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