

that enzymes may be involved in intracellular disulfide interchange or in catalyzing formation of correct disulfide bonds in immunoglobulins, but there is no conclusive evidence of such intracellular activity (14). It seems clear, however, that the blocked L forms discussed in this work, and possibly a variety of other nonproductive intermediates in assembly, can be readily converted to molecular species on the main pathways without enzymatic participation. The apparently small kinetic barriers thus may allow thermodynamic control over the final covalent assembly steps.

In this last connection, the blocked L monomer experiment is interesting and exceptional in that an incompletely assembled intermediate, H₂L, remains in high excess after very long times. A possible explanation is that the released cysteine forms a stable mixed disulfide with H₂L. It is more likely, however, that H₂L is not as reactive as H with L-cysteine. This provides an analogy with the reoxidation system using free L chains in which, as noted above, the second HL bond is formed more slowly than the first. In this view, the driving force for these reactions is the establishment of forms which yield maximal concentrations of tetramer, even if not all disulfide bonds are formed within the tetramer, and the last bond to form, in this case between H₂L and L, may add only marginal stability. The somewhat diminished rate of sulfhydryl disappearance in this experiment is then probably due to the slow rate of oxidation of the cysteine released in reaction 3.

It is hoped that experiments of this kind, coupled with studies of incomplete assembly with the variable half of the L chain (V_L) instead of whole L chain (15), will lead to a more complete understanding of assembly defects and may also allow controlled preparation of various intra- and interspecies hybrids.

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1. The nomenclature used throughout is that recommended by the World Health Organization.
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DNA Synthesis in Cartilage Cells Is Stimulated by Oscillating Electric Fields

Abstract. *External oscillating electric fields (1166 volts per centimeter, 5 hertz) enhanced the incorporation of [³H]thymidine into the DNA of chondrocytes isolated from the proliferative layer of embryonic (16 days) chick epiphysis. Verapamil or tetrodotoxin at 10⁻⁶M concentrations completely blocked the electric field effect. Tetracaine reduced the incorporation of [³H]thymidine in both control and electrically stimulated cells. The findings support the hypothesis that Na⁺ and Ca²⁺ fluxes generated by the electrical perturbation trigger DNA synthesis in these cells.*

Electrical stimuli, applied in various ways, promote appositional and longitudinal bone growth in birds (1), limb regeneration in amphibia (2), and fracture repair in dogs, rabbits, and humans (3). Electric fields (EF's) stimulate proline uptake in cultured fibroblasts (4) and cause dedifferentiation in amphibian erythrocytes (5). Investigation of the mechanism of these effects can contribute to the understanding of epigenetic control of growth and differentiation. In this study we report that oscillating EF's stimulate the incorporation of [³H]thymidine into the DNA of cartilage cells in suspension, and that this effect can be blocked by inhibitors of calcium or sodium fluxes.

Cartilage cells were isolated from the proliferative zone of tibia epiphyses from 16-day chick embryos (6). The cells were incubated for 6 hours in a defined medium at 37°C in the presence of [³H]thymidine and were exposed to a pulsed d-c external EF of 1166 volt/cm, oscillating at 5 hertz. The EF was chosen on the basis of its inhibitory effect on adenosine 3',5'-monophosphate (cyclic AMP) accumulation (7), a condition conducive to cell proliferation in certain systems (8). Under these conditions the EF invariably stimulated [³H]thymidine incorporation, by 53 ± 13 percent (mean ± standard error, 72 experiments, 216 samples and matched controls, range: 24 to 120 percent). The EF-enhanced incorporation of [³H]thymidine occurred in material that could be digested with deoxyribonuclease, whereas the labeling of the material that could be extracted with

trichloroacetic acid was unaffected. The EF effect was first detected after 6 hours of incubation. The lag period suggests that the EF acts on a regulatory step in the cell cycle which precedes DNA synthesis. We also found an increase in DNA synthesis 4 to 6 hours after a brief electrical stimulation (15 minutes).

Skin fibroblasts obtained by collagenase digestion from 10-day-old chick embryos and treated in the same way as the chondrocytes were not stimulated by the EF. Under the same experimental conditions, the EF failed to affect [³H]thymidine incorporation into lymphocytes from rat spleens. However, in bone cells isolated from calvaria of 19-day rat embryos (9), EF's enhanced [³H]thymidine incorporation by 27 ± 1.2 percent (three experiments, 15 samples). Therefore, under the experimental conditions used, the effect appears to be tissue specific.

In formulating a hypothesis for the mechanism of this phenomenon, we took into consideration the similarity of the effects of mechanical and electrical stimuli on bone growth (1, 3) and on cellular cyclic AMP (6, 7, 10) and current theories on the control of cell proliferation (11). Previously, we showed that mechanical stimuli capable of influencing bone growth reduced cellular cyclic AMP in epiphyseal chondrocytes through enhancement of calcium uptake and inhibition of adenylyl cyclase (7). Modulation of ion fluxes and cyclic AMP levels are assumed (12) to be general modes of membrane-mediated cellular communication, which reached evolu-

tionary specialization in neural transmission and hormone action, respectively. The same modes of communication have been invoked in the control of cell division (11). In a variety of organisms ranging from fucoid and sea urchin eggs to lymphocytes and fibroblasts, cell proliferation has been associated with membrane depolarization (13), elevation of intracellular calcium (11, 14) and a drop in cellular cyclic AMP (8).

Electric perturbation of the cell membrane can affect both cyclic AMP and ion fluxes. Polyelectrolytes (15), direct currents through the medium (16), and external EF's (7) alter cyclic AMP accumulation or adenylyl cyclase activity. Effects of electric perturbations on membrane ionic permeability are well documented. Electromechanical forces generated in the cell membrane by rapidly increasing (microsecond) EF's (10^3 volt/cm) can lead to temporary reversible electronic collapse of the membrane (dielectric breakdown). As a result, ions flow along their concentration gradients and alter the membrane potential (17). Pulsating currents of lower intensity can have similar effects (18), also resulting in increased membrane permeability to ions.

We propose that in our experimental system the rapidly increasing potential differences between the plate electrodes briefly perturb the membrane potential either directly (through field effects) or by way of transient currents generated in the electrolyte solution surrounding the cells (19). The changes in membrane potential cause ion fluxes, which are responsible for the EF effects. To test this hypothesis we examined the effect of Na^+ and Ca^{2+} flux inhibitors on EF-stimulated DNA synthesis (Fig. 1). Tetracaine hydrochloride, a local anesthetic which interferes with the Na^+ current in excitable tissues and Ca^{2+} translocation in many cell membranes (20), significantly reduced the extent of ^3H thymidine incorporation in control cells. The inhibition was apparent at a concentration of 10^{-6}M and was dose dependent. The inhibition was partially overcome by the EF at all concentrations of tetracaine examined.

To investigate the contribution of extracellular Ca^{2+} to EF stimulation, we used various Ca^{2+} concentrations. In control cells ^3H thymidine incorporation was 30 to 50 percent higher with 10^{-3}M Ca^{2+} than with a 10^{-6}M concentration; however, at all extracellular Ca^{2+} concentrations between 10^{-8} and 10^{-3}M (21) we observed the same extent of EF stimulation. Verapamil, a Ca^{2+} antagonist which inhibits cellular processes requiring extracellular Ca^{2+} , but was re-

cently also shown to impair sodium influx in myocardial cells (22), abolished the EF effect at a drug concentration of 10^{-6}M .

To elucidate the role of Na^+ ions, we added tetrodotoxin, a specific inhibitor of sodium channels in excitable tissues, to the medium at various concentrations. Tetrodotoxin caused a moderate, dose-dependent reduction in ^3H thymidine incorporation in control cells and a larger reduction in EF-stimulated cells. At 10^{-6}M concentration it completely abolished the EF effect (Fig. 1). Interpretations of these results should take into account the different aspects of the relation between Na^+ and Ca^{2+} : (i) the

effect of membrane Ca^{2+} on Na^+ permeability (23); (ii) the coupling of Na^+ Ca^{2+} fluxes (24); and (iii) the release of Ca^{2+} from intracellular stores, such as sarcoplasmic reticulum, as a result of depolarization (Na^+ influx) (25). The data suggest that the latter mechanism, which is responsible for excitation-contraction coupling in striate muscle, may account for the EF effects on DNA synthesis reported here. Since the phenomenon is not dependent on extracellular Ca^{2+} but is strongly depressed by tetracaine hydrochloride, it appears that membrane depolarization carried by a Na^+ current is followed by an increase in intracellular Ca^{2+} concentration, triggering DNA syn-

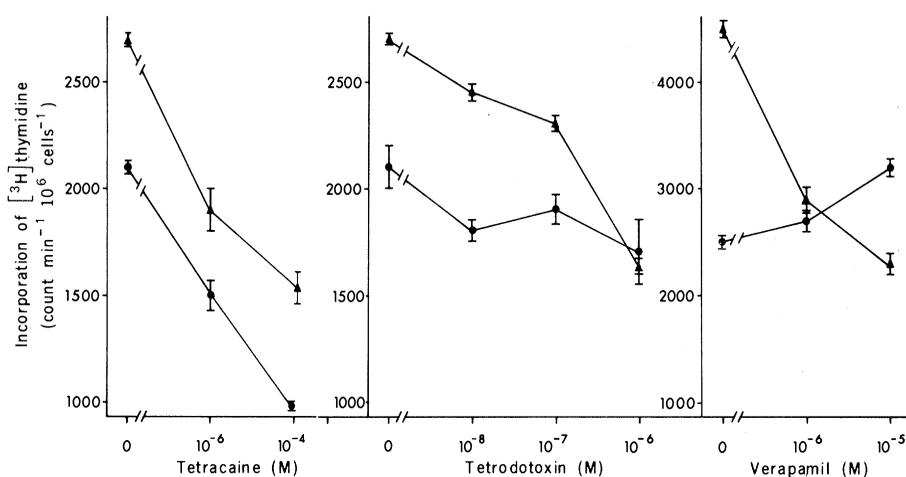


Fig. 1. Effect of tetracaine, tetrodotoxin, and verapamil on ^3H thymidine incorporation into epiphyseal cartilage cells with (▲) and without (●) electric-field perturbation. Experiments were conducted as described in Table 1. Each point represents the mean and standard error of the mean of six samples (two experiments, triplicates).

Table 1. Effect of electric field (EF) on DNA synthesis in epiphyseal cartilage cells. The cells were isolated as described (6, 7); 2×10^6 cells were added to polypropylene culture tubes (17 by 100 mm) containing $0.5 \mu\text{C}$ of ^3H thymidine and 2 ml of minimum essential medium (MEM) with Gey's balanced salt solution buffered with 0.02M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) at pH 7.4 (28). The cells were incubated for the indicated time periods at 37°C in the presence or absence of the EF (29). The incorporation of ^3H thymidine into DNA was measured as described by Ash and Francis (30). The results are the means \pm standard error of the mean of the number of samples shown in parentheses. Matched controls were used in all experiments. Statistical significance was estimated by Student's *t*-test. In one experiment the 6-hour incubation period was terminated by washing the cells with KRG and boiling them. After sonication (15 seconds, 100 watts), the precipitates were treated with deoxyribonuclease ($1 \mu\text{g}/\text{ml}$; bovine pancreas, Worthington). At the time indicated, 1 ml of 5 percent TCA and $100 \mu\text{g}$ of bovine serum albumin were added to 1-ml samples of the incubation mixture. The incorporation of ^3H thymidine was measured as described (30).

Time	^3H Thymidine incorporation (counts min. $^{-1}$ 10^6 cells $^{-1}$)			
	Extractable with TCA		Precipitable with TCA	
	Control	EF	Control	EF
2 hours	20,401 \pm 2,822	20,388 \pm 1,830 (8)	2,541 \pm 165	2,233 \pm 42 (8)
4 hours	15,225 \pm 1,147	15,502 \pm 1,057 (14)	2,634 \pm 167	2,470 \pm 134 (14)
6 hours	19,338 \pm 1,829	21,786 \pm 1,113 (14)	3,274 \pm 248	4,999 \pm 378* (216)
Addition of deoxyribonuclease				
0 minute			2,050	2,496
5 minutes			1,844	2,216
15 minutes			1,608	1,632
30 minutes			468	486

* $P > .001$.

thesis in the subpopulation of epiphyseal cartilage cells susceptible to this stimulation. This hypothesis is consistent with the mode of action of the pharmacological agents used to inhibit the EF effect and with evidence on the control of cell division in other systems.

The findings have further interesting implications. Tetrodotoxin-sensitive "channels" are usually characteristic of cells with fast-spreading action potentials (26). Our data suggest that membrane depolarization may play a role in the control of cell division in epiphyseal chondrocytes. The phenomenon may be related to the embryonic state of the tissue and has been suggested as a possible mechanism for the intercellular communication involved in morphogenesis (27).

Detailed knowledge of the charge distribution and dipole moments in cell membranes (19) and the propagation of EF's in electrolytes would probably add to the understanding of the precise nature of the electrical events experienced by the cells in our system. We have demonstrated that an external oscillating field applied to cartilage cells in suspension generates a perturbation that stimulates DNA synthesis, and that Ca^{2+} and Na^+ fluxes are intimately related to the EF effect. Further studies should show if this phenomenon is involved in the mechanical modulation of bone growth. This information could be used to influence cell proliferation for therapeutic purposes.

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- The EF was generated by two curved copper electrodes, 1.5 cm apart, cemented to the inner surface of a Plexiglas well and connected to a Hewlett Packard 6516A d-c power supply. Polypropylene (1 mm thick) capped test tubes (1.5 cm diameter) containing the cell suspension fitted snugly between the electrodes. The liquid level of the cell suspension and medium approximated the vertical height of the plates. The relative ambient humidity in the incubation mixture was 25 percent (at 37°C). The pulse shape was regulated by a Grass physiologic stimulator (model S4GR). In this study a rectangular-shaped pulse of 1166 volt/cm, 0.1 second on and 0.1 second off was used. The voltage alternated between 0 and 1750 volts. The rise time of the driving voltage was 1850 μ sec.
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Motion of Enclosed Particles Around a Central Mass Point: Errors in the "Apples in a Spacecraft" Model

Alfvén (1) considered the motion of a number of inelastic particles ("apples") enclosed in a spacecraft in circular orbit around a central mass point. He concluded that the system tends toward a final state in which all particles are aligned on the circle described by the center of gravity of the spacecraft. This result was taken by Alfvén as evidence in favor of his hypothesis on the formation of "jet streams" (2).

Unfortunately, Alfvén's reasoning is incorrect and the final state of the system is in reality rather different from what he predicts. This conclusion has apparently escaped notice so far, and Alfvén's result continues to be cited uncritically (3). Thus it appears desirable to correct the record. For convenience I shall designate by "up" and "down" the directions away from and toward the central mass,

respectively. Let r_0 be the radius of the orbit of the spacecraft's center of gravity, and a be the semimajor axis of the elliptical orbit of a particle. If a is less than r_0 , that is, if the particle is, in the mean, "lower" than the spacecraft's center of gravity, its angular velocity is larger than that of the spacecraft. Thus after a while it will hit the front wall of the spacecraft. As a result, the particle loses energy and a decreases. (The mean velocity of the particle on its new orbit will be larger than before; this is the usual paradox of Newtonian mechanics, whereby an artificial satellite losing energy through friction with the atmosphere spirals down to Earth with increasing velocity.) There is then a new collision with the front wall, which decreases a further, and so on. On the other hand, the inelastic collisions tend to damp out the os-