case of the shocks near Crete and in western Turkey the "foreshocks" included shocks with magnitudes as large as 5.9 and 6.2, respectively.

No clear-cut physical explanation has been given for changes in stress axes before strong earthquakes. Brady (*27*) presented theoretical and experimental evidence suggesting that near a concentration of microcracks the axis of compression becomes oriented subparallel to this zone of weakness, but the subsequent events leading to fracture are not well understood.

In summary, a large number of small earthquakes ($M_L \leq 3$) occurred on or near the San Andreas fault near Palmdale, California, between November 1976 and November 1977, constituting an order of magnitude increase in the number of events per year. Although most of the earthquakes clustered tightly in a manner similar to that observed for many foreshock sequences, such clustering is not always diagnostic of foreshocks. The significance of the observed changes in focal mechanism with time is also difficult to evaluate, given the variety of patterns observed in foreshock studies and our incomplete understanding of the processes that precede earthquake faulting. Hence, the implications of the swarm for future large earthquakes on the San Andreas fault are unknown.

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29. We thank D. Anderson, C. Allen, K. Sieh, A. Barrows, C. Johnson, J. Whitcomb, J. Rudnicki, C. Richter, R. Castle, V. Taylor Lehner, D. Tanner, M. French, and L. Blayney for helpful suggestions and technical assistance. This work was supported by grant 14-08-0001-16711 from the U.S. Geological Survey. Contribution No. 3040, Division of Geological and Planetary Sciences, California Institute of Technology.

8 May 1978; revised 10 July 1978

Estrogen-Binding Sites in Endothelial Cell Cultures

Abstract. *The cytosol extracted from a vascular endothelial cell line binds [^{3}H]estradiol with high affinity and a high degree of specificity. In contrast, in experiments performed with cytosol labeled in the intact cell, progesterone and, to a smaller extent, testosterone gave an apparent inhibition of estradiol binding. These data support the concept that ovarian hormones may influence the role of the endothelium in various physiological and pathophysiological conditions.*

The low incidence of atherosclerosis in premenopausal women has suggested that sex-linked factors may have a role in the prevention of this vascular disorder. After menopause, the difference between sexes in the susceptibility to the disease tends to diminish. Similarly, ovariectomized women exhibit a degree of atherosclerosis closer to that of men than healthy women (*1*). Among the changes associated with the loss of ovarian function, variations in the type and amount of circulating blood lipids have been reported (*2*). The possibility that local vascular events that are under the influence of ovarian hormones play a role in the development of the atherosclerotic lesion has not been sufficiently explored. Since an impairment of the endothelial function may be responsible for the changes in structure and composition of the subendothelial intima that are observed during the development of the atherosclerotic plaque, a precise definition of the metabolic characteristics of the endothelium is of particular importance. Experiments performed with intimal tissue may not lead to a correct analysis of the functional activities of the endothelial lining of the artery because, aside from the difficulty of obtaining a pure intimal layer, cells other than endothelial, presumably smooth muscle cells, are present in the intima itself. The availability of an endothelial cell line (*3*) and of a subendothelial cell line derived from the intima of male rabbit aorta has made it possible for us to ascertain whether any of the cell types present in the intima are potentially capable of responding to estrogens. Our results indicate that endothelial cell cultures possess estrogen receptors whereas the other cell line of intimal derivation does not.

The subendothelial cell line used for these studies was obtained by removing first the endothelial lining of the artery as indicated elsewhere (*3*). The cells released into the lumen of the vessel during a second incubation period with enzyme

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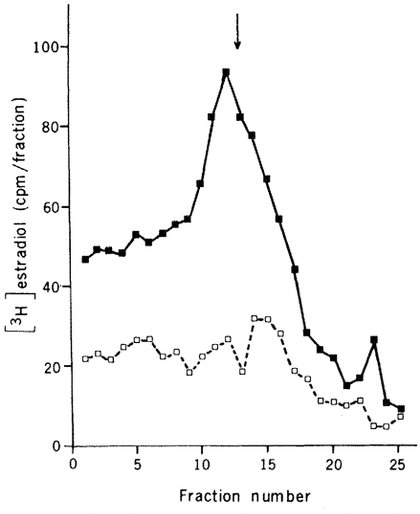
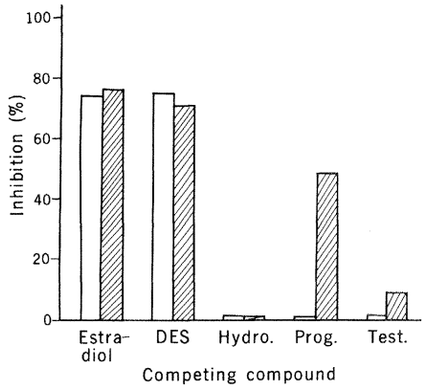
were harvested and plated. A variable amount of endothelial cells may be initially present in this preparation, but they are quickly overgrown by the other cell type whose growth pattern and morphology are strikingly different from those of the endothelial cells; in addition, these cells lack certain properties (blood group antigens, secretion of plasminogen activator) which are present in cultured endothelial cells (*3*).

To prepare the cytosol, we washed the cells twice with phosphate buffered saline, harvested them in the same buffer with a rubber policeman, and then centrifuged the suspension at 400*g* for 3 minutes. The cell pellet was resuspended in 1 ml of cold tris buffer (10 m*M* tris, 5 m*M* EDTA, 2 m*M* β-mercaptoethanol, 0.4*M* KCl, *p*H 7.4). The cells were disrupted with three 10-second bursts of sonication in the cold and the suspension was centrifuged at 100,000*g* for 1 hour at 4°C. The supernatant (cytosol) was used for further experimentation as described below.





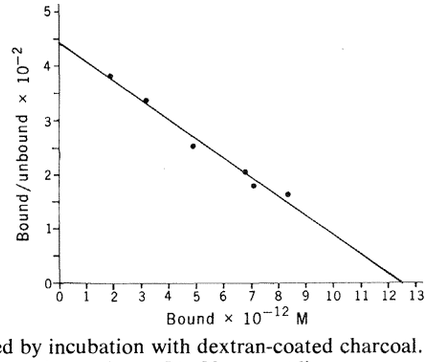


Fig. 1 (left). Sucrose gradient centrifugation of cytosol from endothelial cell cultures labeled with [^{3}H]estradiol. Endothelial cell cultures were incubated in serum-free F-12 for 12 hours at 37°C in a 5 percent CO_2 atmosphere with $10^{-9}M$ [^{3}H]estradiol ([2,4,6,7,16,17-^{3}H(N)]estradiol, 152 Ci/mmole, New England Nuclear) in the presence and in the absence of a 100-fold excess of unlabeled estradiol. Cytosol was prepared as indicated in the text; free and nonspecifically bound estrogen was removed by incubation with dextran-coated charcoal. Samples of 0.3 ml of cytosol (0.8 mg of protein) were layered on a 5 to 20 percent linear sucrose gradient in tris buffer and centrifuged in a Spinco SW 50.1 rotor at 200,000*g* for 16 hours at 2°C. Symbols: ■, cytosol from cells incubated with [^{3}H]estradiol; □, cytosol from cultures incubated with [^{3}H]estradiol in the presence of $10^{-7}M$ unlabeled hormone. The arrow indicates the position of horseradish peroxidase (3.8*S*) added as an internal sedimentation marker. The direction of sedimentation is from right to left. Fig. 2 (top right). Competition with estradiol for estrogen binding sites by various steroids and diethylstilbestrol. Labeled cytosol was obtained by two methods, either by incubating 1-ml portions of cytosol, prepared as indicated in the text, with [^{3}H]estradiol ($5 \times 10^{-10}M$) for 1 hour at room temperature in the presence or in the absence of competing compound ($5 \times 10^{-8}M$) or by extracting it from endothelial cell cultures which had been labeled for 4 hours at 37°C in a 5 percent CO_2 atmosphere in serum-free F-12 medium containing concentrations of hormones similar to those stated above. Incubation with dextran-coated charcoal which removes free and (in part) nonspecifically bound estrogen was performed as stated in the text. Values represent the average of three experiments (*8*). Abbreviations: *DES*, diethylstilbestrol; *Hydro.*, hydrocortisone; *Prog.*, progesterone; *Test.*, testosterone. Open bars, cytosol labeled after extraction from the cell; shaded bars, cytosol labeled in the intact cell. Fig. 3 (bottom right). Scatchard analysis of the binding of radioactive estradiol to endothelial cell cytosol. Portions of cytosol (1.13 mg of protein per milliliter) were incubated for 2 hours at room temperature with increasing concentrations of [^{3}H]estradiol (from $0.5 \times 10^{-10}M$ to $5 \times 10^{-10}M$). The cytosol was then incubated with dextran-coated charcoal and the charcoal was removed by centrifugation; a portion of the supernatant was placed in toluene [toluene, butyl 2-phenyl-5-(4-biphenyl)-1,3,4-oxadiazole, 9 g/liter, and BBS3 10 percent, Beckman] and the radioactivity was measured in a Beckman liquid scintillation spectrometer. The dissociation constant, K_d, was $2.8 \times 10^{-10}M$ and the number of binding sites per milligram of cytosol protein was 11×10^{-15} moles. The number of binding sites may represent a minimum value because of the high concentration of dextran-coated charcoal (to remove free and nonspecifically bound steroid) used in our experimental system in order to obtain an accurate binding constant. No attempt was made to define optimum culture conditions for the determination of the number of receptors.

Incubation with dextran-coated charcoal was carried out as follows: 1 ml of cytosol was added to 0.2 ml of dextran-coated charcoal suspension [7.2 g of Norit A and 0.72 g of dextran T-80 (Pharmacia) in 100 ml of tris buffer] and the suspension was incubated for 20 minutes in ice with intermittent shaking. The charcoal was removed by centrifugation. The protein content of each cytosol preparation was determined by the procedure of Lowry (*4*). In all experiments, cultures were used at near confluence.

Evidence for the presence of estrogen receptors in endothelial cell cultures was obtained by determining the rate of migration of bound [^{3}H]estradiol through a sucrose density gradient (Fig. 1). The receptor to which the estrogen is bound has an *S* value of 4.1, and the binding is specific since the amount of [^{3}H]estradiol bound in the 4*S* area is very reduced by excess concentration of unlabeled hormone (*5*).

Only estrogens competed with the tritiated form of estradiol for the specific binding sites: none of the other steroid hormones tested was capable of displacing labeled estradiol in experiments performed with cytosol labeled after extraction from the cell (Fig. 2). However, in experiments performed with cytosol labeled in the intact cell, progesterone, and to some extent testosterone, appeared to inhibit binding of estradiol to the receptor (Fig. 2); these results could be explained either by the fact that the two steroid hormones decrease the number of estrogen receptors available [as has been shown for progesterone (*6*)] or by their conversion to a derivative capable of competing with estradiol for the receptor binding site. Competition with estradiol for transport into the cell is a possibility that should perhaps be considered, although the entry of estrogen into its target cells is classically thought of as a simple diffusion process. These findings are of particular interest in view of the fact that the blood levels of female or male hormones may, under certain conditions, be sufficiently high to compete effectively with sex hormones administered for therapeutic or experimental purposes. The Scatchard (*7*) plot (Fig. 3) of the binding phenomenon indicates that the binding is of high affinity; the number of binding sites has been found to be 11×10^{-15} moles per milligram of cytosol protein. It is conceivable that, as has been suggested for other functional activities of the endothelium (*3*), the number of binding sites may differ in endothelial cell cultures derived from different vascular beds. No specific binding activity for estradiol was found to be

present in the other intimal cell line: under conditions similar to those described in Fig. 2 for experiments performed with cytosol labeled after extraction from the cell, inhibition by estradiol was 6 percent, by testosterone 5 percent, and by hydrocortisone 6 percent.

The vascular endothelial cell line used for these studies possesses an array of receptors for various hormones and neurotransmitters (*3*), a finding which suggests that, through their complex and finely regulated metabolic activity, endothelial cells play an essential role in the regulation of local circulation. In this respect, it is worth noting that the earliest detectable effect of estrogens on the uterus is increased blood supply and increased permeability of the vascular wall (*1*). Thus, the presence of estrogen receptors in endothelial cells seems to suggest that the response of the blood vessels of target organs to estrogens may be an important aspect of the mechanism through which these hormones regulate the cyclical functional variations of the reproductive system. As already noted, clinical observations suggest that the presence of a normal level of circulating ovarian hormones coincides with a lower degree of susceptibility to atherosclerosis. The finding that endothelial cells are potentially capable of responding to estrogens may stimulate attempts to elucidate the nature of the endothelial function which is regulated by these hormones; with this knowledge, one would be better able to evaluate which role a normal hormonal balance may play in delaying the development of the atherosclerotic lesion.

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5. Experiments involving the labeling of endothelial cell cultures with [^{3}H]estradiol for various lengths of time over a 3-hour period, and the separate extraction of the labeled receptor from the cytoplasm and from the nucleus, show that the amount of labeled receptor which is extractable from the nucleus increases with time. During the same period of time, the amount of labeled receptor in the cytoplasm tends to decrease. These results are consistent with the concept that the receptor-hormone complex forms in the cytoplasm and then appears in the nucleus.
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8. Percentage of inhibition was determined by dividing the value of radioactivity bound to cytosol labeled in the presence of competing compound by the value obtained with cytosol labeled in the absence of competing hormone multiplied by 100. Percentage of inhibition is a measure of the fraction of specifically bound [^{3}H]estrogen which is displaced by unlabeled hormone. In the case when the competing compound is unlabeled estradiol, percentage of inhibition also represents the amount of radioactivity which is bound to specific estrogen receptors. The estradiol molarities used for these experiments slightly exceeded the level of hormone at which all the receptor sites are saturated. At higher estradiol molarities the amount of radioactivity which is not specifically bound increases because the level of estradiol at which all the receptor sites are saturated is largely exceeded. Therefore, the fraction of radioactive estradiol displaced by the unlabeled estrogen varies with the concentration of radioactive estradiol in the medium, being higher when the radioactive estradiol concentration in the incubation medium is lower.
9. This work was supported by NIH grant HL 17995.

14 February 1978; revised 25 April 1978

Trisodium Phosphonoformate, a New Antiviral Compound

Abstract. *Trisodium phosphonoformate selectively inhibits cell-free DNA polymerase activity induced by herpesvirus. The new inhibitor has an antiviral effect on herpes simplex virus types 1 and 2, pseudorabies virus, and infectious bovine rhinotracheitis virus in cell culture. It has a good therapeutic activity against cutaneous herpes simplex virus infection in guinea pigs.*

The search for antiviral agents has resulted in the discovery of compounds with activity against herpes simplex virus (HSV) infections, but only a few of these compounds have been submitted to clinical use. Herpes keratitis has been treated with idoxuridine (5-iodo-2′-deoxyuridine) and vidarabine (9-β-D-arabinofuranosyl adenine) (*1*, *2*). Herpes zoster (*3*) and herpes encephalitis (*4*) respond to treatment with vidarabine. However, the high cytotoxicity (*5*, *6*) of these compounds limits their usefulness. A less cytotoxic compound, phosphonoacetic acid (PAA), specifically inhibits DNA polymerase from different herpesviruses (*7*) and arrests herpesvirus infection in mice (*8*) and guinea pigs (*9*), but no clinical results with PAA have been reported.

As pointed out by Cohen (*10*), a rational approach to viral chemotherapy should involve attempts to design compounds that interact specifically with the viral enzymes leaving the cellular enzymes unaffected. As part of a screening program for antiviral drugs, we have used cell-free HSV-1 DNA polymerase and influenza RNA polymerase and, as controls, cellular DNA and RNA polymerases to select specific HSV DNA and influenza RNA polymerase inhibitors (*11*).

One series of compounds investigated included analogs of pyrophosphate, which may interact with the binding of nucleoside triphosphates to polymerases (*12*). One of the first substances tested in this program was the trisodium salt of phosphonoformic acid (PFA), a compound originally synthesized in 1924 by Nylén (*13*) and structurally related to PAA. The crystal structure of this salt has been determined (*14*).

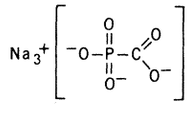

Table 1 shows the effect of PFA and, for comparison, PAA on several DNA and RNA polymerases. It is evident that HSV-1 DNA polymerase is inhibited at low concentrations by both PFA (*14a*) and PAA, and that the two compounds have comparable activities against several DNA polymerases. The preferential inhibition of HSV-1 DNA polymerase in

Table 1. Inhibition of cell-free polymerase activity

Polymerase	Concentration giving 50 percent inhibition (μ*M*)	
	PFA	PAA
HSV-1 DNA polymerase*	3.5	7.0
DNA polymerase α, calf thymus (25 U per assay)†	50.0	75.0
DNA polymerase α, calf thymus (2.5 U per assay)†	3.5	6.5
Micrococcus luteus DNA polymerase†	>500.0	>500.0
Escherichia coli DNA polymerase I†	>500.0	>500.0
Influenza RNA polymerase‡	20.0	300.0
RNA polymerase I, calf thymus†	>500.0	>500.0
RNA polymerase II, calf thymus†	>500.0	>500.0
Escherichia coli RNA polymerase	>500.0	>500.0

*Prepared and assayed according to Weissbach *et al.* (*21*). †Obtained and assayed as described earlier (*22*). ‡Prepared and assayed as described earlier (*22*), but Mn^{2+} was not used in the assay.

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