hormone on the labeling of this protein continued to increase as a function of development. After the completion of metamorphosis (stage 66 and thereafter), estrogen evoked the selective synthesis of vitellogenin without altering the labeling of other exported proteins.

For a more critical test for the presence of vitellogenin, immunoprecipitates were dissolved in sodium dodecyl sulfate–sample buffer and then subjected to electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. The resulting distributions of ^{35}S-labeled peptides in the precipitates from media of liver explants from tadpoles at stages 52, 54, and 59 are shown in the fluorographs of Fig. 2. Only the explants from stages 54 and 59 that were cultured in the presence of estrogen contained ^{35}S-labeled proteins that comigrated with authentic vitellogenin marker, and this was the major ^{35}S-labeled protein in these immunoprecipitates. The minor amounts of other peptides in the immunoprecipitates probably result from entrapment. Figure 2 also shows fluorographs of gels containing ^{32}P-labeled proteins from unfractionated culture media of liver explants from adults. A single phosphoprotein that comigrated with the vitellogenin marker was present only in the culture media of estrogen-treated explants; this confirms studies (*2*) that indicate that vitellogenin is the only phosphoprotein secreted by hepatocytes from adult *X. laevis*. We have not yet been successful in labeling vitellogenin in tadpole liver explants with ^{32}P.

Double-labeling experiments and steroid specificity experiments (*11*) indicate that estradiol does not affect the rate of precursor uptake and that the observed responses are specific for estradiol-17β. The vitellogenic response is specific for estrogen in the explant system described in (*5*).

Accompanying the estrogen-induced synthesis of vitellogenin in adult hepatocytes are stimulations of other cellular components or activities. Among these are an acceleration of general protein synthesis (*12*), a net increase in ribosomes (*13*), a massive proliferation of endoplasmic reticulum (*13, 14*), and an increase in the activity of isolated ribosomes (*15*). It has been suggested (*14*) that the coordination of these cellular responses facilitates the translation and posttranslational processing of vitellogenin. Whether these coordinated responses are linked inseparably to vitellogenin synthesis or can occur by separate and independent pathways remains unknown.

One interpretation of our results is that during the differentiation of frog hepatocytes, the vitellogenin genes are either not responsive or only partially responsive to estrogen at early stages, when other coordinated responses to the hormone occur and consequently facilitate the synthesis and perhaps the export of other secreted proteins (see Table 1 and Fig. 1). We suggest that these coordinated responses to estrogen are separable from the end-point response in liver explants from tadpoles, since marked effects of the hormone on labeling of exported proteins were observed in the absence of estrogen-induced synthesis of vitellogenin at stage 53 (see Table 1 and Fig. 1A). It is also possible that the synthesis of vitellogenin was induced at this stage but that vitellogenin was not exported from the hepatocytes. We consider this unlikely, because other proteins were exported.

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Phase Resetting and Annihilation of Pacemaker Activity in Cardiac Tissue

Abstract. *Spontaneous rhythmic activity in isolated cardiac pacemaker cells can be terminated by a brief, subthreshold, depolarizing or hyperpolarizing perturbation of the proper magnitude applied at a specific point in the pacemaker cycle. Evidence is provided in support of a topological theory of the existence of a "singular" point in cardiac oscillators.*

The study of the dynamic behavior of cardiac pacemaker cells in response to discrete perturbations has provided important clues about the mechanisms of more complex interactions between the pacemakers and their normal surroundings (*1*). Studies in which various types of cardiac tissues were used (*2, 3*) have demonstrated that phase shifts occur in response to brief, subthreshold, depolarizing or hyperpolarizing stimuli. Phase-response curves have been constructed for these stimuli and used to predict the entrainment behavior of cardiac pacemaker cells when interacting with such inputs as brief vagal stimuli or electrotonic potentials across an area of depressed excitability.

Not surprisingly, the results of those studies have shown that many similarities exist between the characteristics of cardiac pacemakers and of other biological oscillatory systems. The use of perturbation techniques for the analysis of the dynamic behavior of a variety of periodic systems has demonstrated that even though the underlying cyclic mechanisms may be vastly different, they all share common behavioral patterns when interacting with their environment (*4–7*). Topological techniques have also been used in the context of pacemaker activity and have shown interesting analogies to other periodic systems. Through his studies of circadian rhythms in fruit flies and oscillatory glycolysis in yeast, Winfree (*8*) developed a theory involving phase resetting patterns of oscillatory systems. He suggested that the features that evolve from his theory are not limit-

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ed to biochemical and circadian oscillators but can be demonstrated in neuronal pacemakers as well (*9*).

Winfree's analysis involves the application of perturbing stimuli at different points in the pacemaker cycle. His theory, in which techniques of differential topology are used, predicts that if the phase resetting of the pacemaker in response to the perturbation follows certain specific patterns, then there must be a characteristic stimulus magnitude and timing at which pacemaker activity is completely annihilated.

The applicability of Winfree's theory to neuronal pacemakers has been confirmed both theoretically and experimentally in the squid axon membrane. Best (*10*) used the Hodgkin-Huxley equations for squid axon membranes with a current bias to induce rhythmicity. He applied depolarizing and hyperpolarizing current clamps at various times during the spontaneous cycle of the simulated pacemaker, and demonstrated that a stimulus of a certain amplitude at a critical phase in the cycle terminates the pacemaker rhythm. Spontaneous activity recovers only after a few cycles of subthreshold oscillation, or it can be restored by the application of a second brief pulse of large intensity. Guttman *et al.* (*11*) provided experimental proof that repetitive firing in clamped squid axons bathed in weak calcium solutions can be stopped by a brief perturbation of the proper magnitude applied at a specific position in the spontaneous cycle. We now report that, under certain experimental conditions, pacemaker activity in spontaneously beating cardiac tissues can also be terminated by the application of a brief, subthreshold, depolarizing or hyperpolarizing stimulus applied at a critical point in the pacemaker cycle.

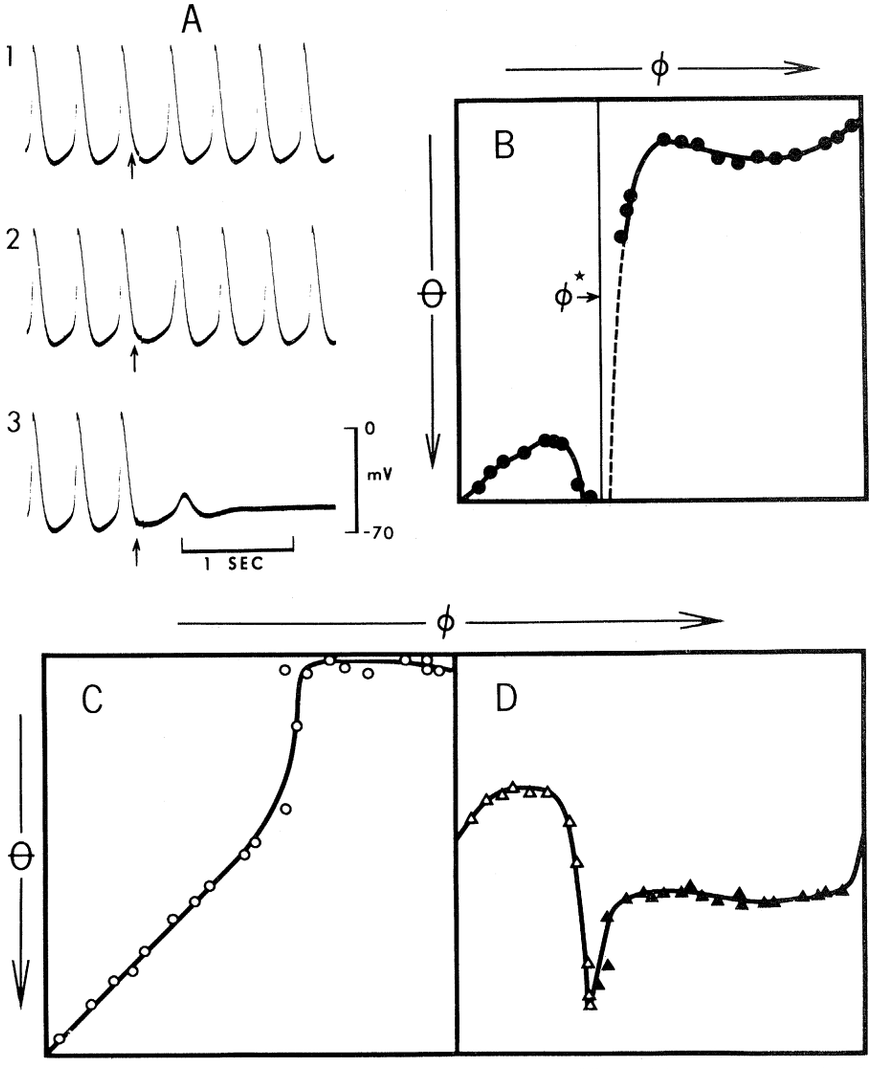

Fig. 1. Phase resetting and cessation of spontaneous activity of SA nodal pacemakers by brief, subthreshold, depolarizing current pulses. (A) Analog microelectrode recordings from a kitten SA nodal strip mounted in the sucrose chamber. (B to D) Cophase (Θ) plots, equivalent to Winfree's (*8*) new phase-old phase, at three different stimulus durations in a different SA nodal preparation. (B) Changes effected by a current pulse of intermediate duration (30 msec) where, ϕ^* is phase at which activity ceased (singularity phase). (C) Current pulses of briefer duration (10 msec) induced a type 1 resetting curve. (D) Current pulses of longer duration (50 msec) yielded a type 0 resetting curve.

Strips of sinoatrial (SA) nodes from kittens and Purkinje fibers from dogs and calves were prepared as described elsewhere (*1, 3*) and were mounted in a three-chambered tissue bath. After an equilibration period of 1 hour, the central chamber was filled with an isotonic sucrose solution (*12*), the test chamber with Tyrode's solution containing 4.0 m*M* KCl and the third chamber with Tyrode's solution containing 20 m*M* KCl. Spontaneous activity was constantly monitored in the test chamber by recording transmembrane potentials differentially with two KCl microelectrodes (d-c resistance, 10 to 40 megohms) and by displaying the amplified signals on an oscilloscope. In all experiments the upstroke of the action potential recorded from the pacemaker cell in the test chamber was used to trigger a digital stimulator after various delays. The stimulator provided a current pulse of variable duration and either polarity every 10 to 20 seconds, delivered through Ag-AgCl electrodes in the outer chambers.

We have shown elsewhere (*1, 3*) that, in general, scanning the pacemaker cycle with brief perturbations of either polarity may result in abbreviations or prolongations of the immediately affected cycle, depending on the timing. In most preparations these changes were short-lived and resulted in phase shifts of the subsequent discharges with respect to the control cycle, but they did not alter the pacemaker cycle permanently. In some experiments, however, we found that spontaneous activity in cardiac tissues could be stopped with subthreshold depolarizing or hyperpolarizing current pulses scanning the pacemaker cycle. The three traces in Fig. 1A are transmembrane potentials recorded from the same SA nodal cell in the test compartment of a sucrose gap preparation. The stimulus was a 50-msec, subthreshold, depolarizing current pulse (4.2×10^{-6} A) applied across the sucrose gap at different phases (ϕ's). As the stimulus was applied progressively later (arrows), increasing delays were produced (Fig. 1, A1 and A2). At ϕ = 130 msec (A3), the current pulse was followed by an incomplete repolarization, highly damped subthreshold os-

cillations, and complete suppression of pacemaker activity.

Composite results of a more complete experiment are plotted in Fig. 1, B to D. The data are presented (after Winfree's format) as cophase (Θ) versus phase (ϕ), where Θ represents the interval between the initiation of the current pulse and the start of the next discharge (whether delayed or advanced). In each plot, ϕ increases to the right through the spontaneous cycle of about 570 msec and Θ increases downward, also through one cycle of 570 msec. Figure 1B shows that as the pacemaker cycle was scanned with 30-msec current pulses (12×10^{-6} A), early stimuli delayed the next discharge and resulted in large Θ's. When the stimulus was applied at 35.8 percent of the spontaneous cycle (vertical line in Fig. 1B), pacemaker activity was stopped (rhythmicity was restorable by a current pulse). If the same stimulus was applied at a later phase, it simply reset the phase of subsequent discharges. When the duration of the stimulus was decreased to 10 msec (Fig. 1C), Θ decreased progressively as ϕ increased, resulting in a resetting curve with an average slope of 1 (type 1 resetting). In contrast, when the stimulus duration was increased to 50 msec (Fig. 1D) and the individual points were centered about the horizontal axis (*8*), the data resulted in a curve with an average slope of 0 (type 0 resetting). This type 0 resetting is critical, as indicated by Winfree (*8, 9*). It is the means by which singular behavior at shorter pulse durations can be foreseen.

As Winfree's theory suggests, the complete data points of Fig. 1, B to D, could be plotted in a single three-dimensional graph by adding the current pulse duration (S) as a third axis. A smooth, two-dimensional "resetting surface" through these Θ (ϕ, S) data would resemble a vertical corkscrew with its rotation axis lying at 204 msec (35.8 percent of pacemaker cycle) and S^* at 30 msec.

The effects of brief hyperpolarizing current pulses on SA nodal pacemaker activity are also dependent on their timing (*2*). In some experiments, not only did the hyperpolarizing input phase-shift subsequent discharges (Fig. 2, B and C), but a stimulus of proper magnitude, duration, and timing completely abolished pacemaker activity in this SA nodal preparation (Fig. 2D). Similar responses were recorded when dog and calf Purkinje fiber pacemakers were used.

These findings are qualitatively similar to the theoretical results for the standard Hodgkin-Huxley equations (*10*) and to the experimental results reported by Guttman *et al.* (*11*). This response pattern can probably be generalized to all types of cardiac pacemakers and should also be demonstrable in computer simulations, using modified Hodgkin-Huxley equations for cardiac Purkinje fibers (*13*) and for ventricular muscle cells (*14*).

The mechanisms behind these phenomena have been explained in terms of repetitive firing of the Hodgkin-Huxley model at various levels of membrane depolarization. It has been shown that for certain ranges of depolarizing current intensity, the equations contain a stable "singular" point in addition to the stable limit cycle of spontaneous pacemaker activity. By experimentally plotting the phase plane trajectory (*15*) of the squid axon in two dimensions, Guttman *et al.* (*11*) have shown that with a brief current clamp of the appropriate intensity and timing, the preparation can be forced off the stable limit cycle and into the domain of attraction of the singular point.

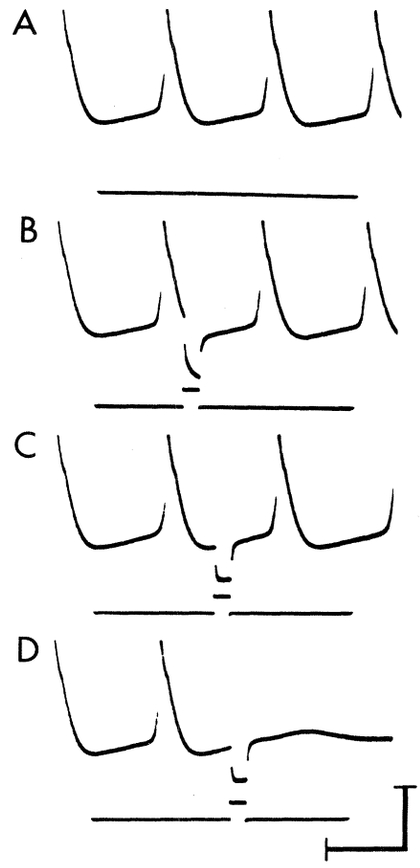

Fig. 2. Suppression of pacemaker activity of an SA nodal cell by a brief, hyperpolarizing current pulse. A kitten SA nodal strip mounted in the sucrose chamber was used. (A) Control recording; mean pacemaker cycle length, 640 msec. (B) A 100-msec, 6.4×10^{-6} A, hyperpolarizing pulse applied at an early phase accelerated the next discharge. (C) A pulse of the same duration, but with magnitude and polarity applied at a later phase, slightly delayed the next discharge. (D) A similar pulse delivered at about 67 percent of the spontaneous cycle induced a hyperpolarization that was followed by damped subthreshold oscillations and by complete suppression of spontaneous activity. Calibrations: vertical, 50 mV and 2.5×10^{-5} A; horizontal, 500 msec.

The demonstration of the coexistence of the stable limit cycle with the singular point and the determination of their positions in time and space with the aid of the topological techniques of oscillator theory (*8*) should provide additional clues to the mechanisms of pacemaker activity in cardiac tissues. They should also broaden our understanding of the characteristic behavior of cardiac pacemakers when they interact with their natural environment under normal or abnormal circumstances. For example, although the inhomogeneous distribution of vagal fibers in the SA region of the heart (*16*) suggests that it is not likely that a single vagal discharge can completely suppress spontaneous pacemaker activity in this region, phase resetting behavior of the individual pacemaker cells should ultimately determine the nature of the overt response to the vagal perturbation.

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