central part of the chondrophore. The surface of nymphal and right chondrophoral prisms in contact with the ligament is finely granular in appearance (Fig. 2A). Fractures of these ligostracal layers expose bundles of calcified fibers clinging to the sides of the prisms (Fig. 2B).

The finely tuberculous, pitted, spaced arrangement of the distal ends of prisms in the oyster ligostracum strongly suggest that the principal function of this layer is to bind the ligament to umbonal shell. The presence of aragonitic fibers [characteristic of the resilial ligament (*13*)] deep among the prisms (Fig. 2B) supports this conclusion. The ligostracum of the hinge is analogous to the mosaicostracum, which binds the periostracum to the valves (*1*, *2*). We suggest that the functional term ligostracum replace the term mosaicostracum and be used to describe all thin mineralized layers that bind external organic layers, such as the ligament and periostracum, to the underlying mineral shell.

Muscle attachment sites (myostracal prisms) in mollusks are invariably aragonitic (*8*). With the exception of the chondrophore in the left valve of oysters, which often contains calcite, all ligostracal layers we examined were aragonitic (Table 1). It would thus appear that aragonitic prisms may serve the function of attachment of muscular and other organic layers better than calcitic shell units.

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Xenopus Liver: Ontogeny of Estrogen Responsiveness

Abstract. *Estradiol-17β stimulates the synthesis of numerous proteins exported into the culture medium by* Xenopus *tadpole liver tissue obtained after stage 50 and throughout metamorphosis to stage 66. Although estrogen-induced vitellogenin can be detected as early as stage 54, it is a minor percentage of the exported proteins until after the completion of metamorphosis. In hepatic tissue obtained after metamorphosis, the hormone evokes the synthesis of vitellogenin specifically without affecting the labeling of other secreted proteins.*

In parenchymal cells of the liver of the African clawed frog *Xenopus laevis*, estradiol-17β (1,3,5-estratriene-3,17β-diol) evokes a well-defined end-point response, the synthesis de novo of vitellogenin, which is the precursor of the major egg-yolk proteins (*1*). Although this estrogen-induced response occurs naturally only in sexually mature females, the normally dormant vitellogenin genes of the adult male hepatocytes can be activated by exogenous estrogen (*2*). Hence, the hepatic vitellogenin genes of male *X. laevis* frogs are not permanently inactivated as a result of differentiation, and the cellular components required for the actions of this female sex hormone are present in their livers. But, when do hepatic cells acquire the ability to respond to estrogen during the course of their development? Identification of responsive and nonresponsive hepatocytes

Table 1. Effect of estradiol-17β on incorporation of ^{35}S-labeled methionine into exported proteins in culture media by liver explants of *X. laevis*. Tadpoles (stages 48 to 65) and juvenile frogs (stage 66 and older) were obtained by gonadotrophin-induced matings of adult frogs. The stage of development of each tadpole was determined by using the method of Niewkoop and Faber (*16*). For each experiment, the livers from 30 to 80 tadpoles at the same stage of development were excised under sterile conditions, pooled in sterile culture medium, and divided into two groups. One group was cultured in the continuous presence of estradiol-17β (10^{-8}*M*). To the control group, an equal volume (10 μl) of the hormone solvent propylene glycol was added. [The presence or absence of estradiol-17β is indicated by (+) or (−), respectively.] Livers from juvenile and adult frogs were processed in a similar manner, except that they were cut into 1-mm cubes before being cultured. After culture for 7 days with daily changes of the media (2 ml per group), the hepatic explants were placed in fresh medium containing ^{35}S-labeled methionine (Amersham Corp.; initial specific activity, 250 to 935 Ci/mmole) to label the proteins. The isotope was added to give a final specific activity of 4 Ci/mmole. The culture medium and the conditions for the culture and for the labeling of proteins were the same as those described by Wangh and Knowland (*5*). After a 7-hour incubation, the media were centrifuged at 1000*g* for 15 minutes to remove free cells. Triplicate portions (100 μl) of each medium (*5*) were taken from each culture to determine acid-insoluble incorporation into the secreted proteins (*17*).

Source of explant		Treatment [estradiol-17β (10^{-8}*M*)]	[^{35}S]Methionine incorporation (count/min per 100 μl)*	Percent of control value
Tissue	Developmental stage			
Liver	49 to 50 (tadpole)	+	2,507 ± 262	102
		−	2,457 ± 415	
Liver	51 to 52 (tadpole)	+	32,557 ± 4,887	307
		−	10,571 ± 2,183	
Liver	53 (tadpole)	+	49,922 ± 7,259	594
		−	8,404 ± 2,212	
Liver	54 to 55 (tadpole)	+	11,272 ± 742	299
		−	5,649 ± 75	
Liver	58 to 59 (tadpole)	+	35,350 ± 6,399	1,125
		−	3,141 ± 540	
Heart	53 (tadpole)	+	0	
		−	0	
Eye	53 (tadpole)	+	410 ± 132	96
		−	424 ± 116	
Liver	66 + 7 days (juvenile frog)	+	29,457 ± 1,308	164
		−	17,947 ± 978	
Liver	66 + 4 days (juvenile frog)	+	6,102 ± 175	174
		−	3,494 ± 122	
Liver	66 + 4 days (juvenile frog)	+	10,545 ± 620	180
		−	5,830 ± 386	
Liver	Adult	+	27,642 ± 1,863	258
		−	10,710 ± 594	
Liver	Adult	+	5,784 ± 334	311
		−	1,856 ± 176	
Liver	Adult	+	29,780 ± 1,367	183
		−	15,352 ± 457	

*Mean ± standard deviation.

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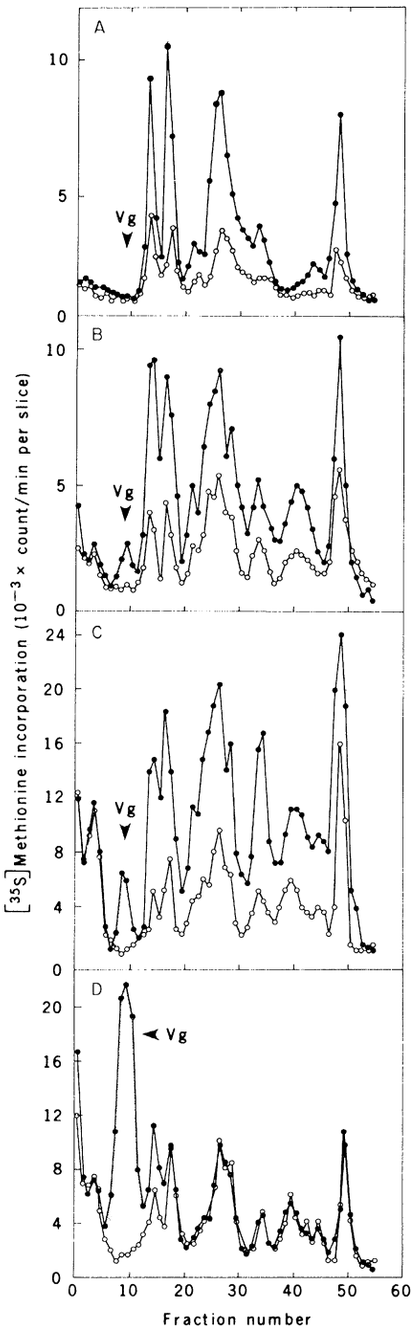
as a function of development should permit biochemical comparisons of such cells and thereby help to elucidate the underlying molecular changes that give these cells the capacity to respond to estrogen.

Follett and Redshaw (*3*) reported qualitative, immunological evidence indicating that hepatic cells of *X. laevis* attain the ability to respond to estrogen only after the completion of metamorphosis at stage 66. More sensitive methods for the analysis and detection of labeled proteins have since been developed (*4*); furthermore, it has been shown that estrogen evokes vitellogenin synthesis in hepatic explants from mature *X. laevis* of either sex (*5, 6*). Therefore, we have reexamined the ontogeny of responsiveness to estrogen in hepatic tissue by using the explant-culture system (*5*). Because vitellogenin is secreted soon after its synthesis (*7*), it accumulates in the culture medium of the explants provided that labeling periods are of sufficient duration (*5, 6*). To test explants for their capacity to respond to estrogen, we accordingly confined our analyses to the labeled proteins in the culture medium.

An unexpected finding was that estrogen stimulated the labeling of most proteins exported into the culture medium by tadpole liver explants at early developmental stages (51 to 53) when no vitellogenin synthesis was evoked by the hormone. The same phenomenon was also observed at developmental stages 54 to 65, stages at which vitellogenin was at most a minor component of the exported proteins. In marked contrast, hepatic tissue explanted after the completion of metamorphosis (stage 66 and thereafter) had a much greater capacity to synthesize vitellogenin in response to the hormone; furthermore, the induced synthesis of vitellogenin accounted for nearly all of the estrogen-enhanced labeling of the exported protein (see Fig. 1). Our data indicate that estrogen responsiveness (as defined by estrogen-evoked synthesis of vitellogenin) occurs earlier (at stage 54) than previously reported (*8, 9*). Nevertheless, our findings agree with those reports in that responsiveness to the hormone increased progressively during metamorphosis, and they are consistent with evidence (*9*) that thyroxine is required to establish this capacity in developing frog liver.



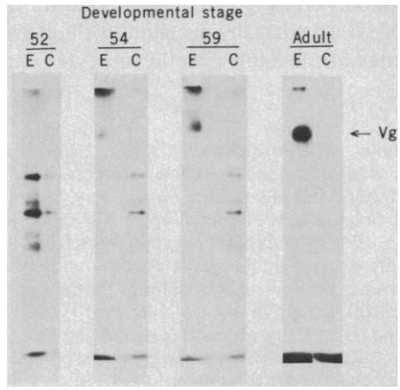


Fig. 1 (left). Electrophoretic distribution of [^{35}S]methionine-labeled proteins in culture media of hepatic explants. Samples of culture media from hepatic explants were obtained as described in the legend to Table 1. The discontinuous gel system of Laemmli (*18*) was used; the resolving gel and the stacking gel were 7.5 and 3.5 percent acrylamide, respectively. After electrophoresis, the gels were cut into 2-mm slices and the radioactivity in each was measured with an efficiency of 81 percent (*13*). Shaded regions indicate estrogen-induced vitellogenin and *Vg* indicates the position of authentic vitellogenin standard. Results are shown for (A) stage 53, (B) stage 54, (C) stage 59, and (D) adult explants. (●) Media from explants cultured in the presence of estradiol-17β; (○) media from explants cultured in the absence of estradiol-17β. Fig. 2 (right). Fluorographs of immunoprecipitable, exported proteins separated by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. Equal amounts of tissue, obtained at the indicated stages, were cultured in equal volumes of media as described in the legend to Table 1. Equal portions of the media (500 μl) were immunoprecipitated and prepared for electrophoresis (*13*). After electrophoresis, the slab gels were processed and exposed to x-ray film (*4*) for 7 days. The fluorographs show samples cultured in the presence of estradiol-17β (*E*), samples cultured in the absence of the hormone but in the presence of the hormone solvent (*C*) (see Table 1), and the gel position of authentic vitellogenin marker (*Vg*).

Table 1 shows the effects of estrogen on the incorporation of ^{35}S-labeled methionine into the proteins in the culture medium of hepatic explants obtained at the indicated stages. Estrogen stimulated the labeling of secreted hepatic proteins, the earliest developmental stages in which this response occurred were tadpole stages 51 and 52, and this response tended to increase between stages 51 and the latter stages of metamorphosis (*10*). Nontarget tissues such as heart and eye displayed no effects of estrogen on the labeling of exported proteins.

To further investigate the identity of these labeled proteins and to make quantitative comparisons, equal portions of the medium were subjected to electrophoresis in polyacrylamide gels. Distributions of the labeled proteins at selected but representative stages of development are shown in Fig. 1. Explants treated with estrogen at stages 51 to 53 revealed no peaks of vitellogenin but, unexpectedly, their exported proteins were labeled to a greater extent than those of control samples. At stage 54 we detected a minor but distinct estrogen-induced peak with an electrophoretic mobility corresponding to that of vitellogenin (molecular weight $\simeq$ 200,000). Estrogen continued to enhance the labeling of not only this presumptive vitellogenin peak but also that of other exported proteins to stage 66. We emphasize that the presumptive vitellogenin peak in all samples from estrogen-treated livers from stage 54 to 65 was only a minor component, even though the effect of the

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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