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A Phase-shift Model for the Spatial and Temporal Organization of Developing Systems†

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The structure of any differentiated tissue results from a well-defined sequence of events in which the spatial and temporal organization of the developing tissue mass are intimately related. It is as though every cell has access to, and can read, a clock and a map (Wolpert's positional information). A model developed in the present paper is one in which the map arises from wave-like propagation of activity from localized clocks or pacemakers. Individual cells are supposed temporally organized in the sense that biochemical events essential for the control of development recur periodically. This temporal organization of an individual cell is converted by functional coupling between cells into a spatial ordering of the temporal organization. More explicitly a periodic event is postulated which propagates outward from a pacemaker region, synchronizing the tissue and providing a time base for development. Intercellular signalling, entrainment of all cells in the tissue by the fastest cells in the pacemaker region, and a refractory period to guarantee unidirectional propagation are the essential features of the propagation; they permit the derivation of a wave equation and a set of boundary conditions. An underlying gradient of frequency of the event establishes the position of the pacemaker region and the sense of propagation. A second event which propagates more slowly than the first provides positional information in the form of a one-dimensional sequence of surfaces of constant phase difference between the two events. A third event is used to regulate the pattern of phase difference and thus establish size-

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independent structures. The longest trajectory orthogonal to the surfaces of constant phase difference beginning at the pacemaker region and terminating at the regulating region defines a developmental axis of definite polarity. The model is readily extended to more than one axis, i.e. multi-dimensional positional information. It has a high informational capacity and is readily applied to the discussion of particular developmental phenomena. To illustrate its utility, we discuss development and regeneration in *Hydra*, positional in the early amphibian embryo, and the retinal-neural tectal projection of the amphibian visual system. Specific experiments to test for the existence of the postulated periodic events and their consequences are suggested. Some preliminary experimental results on *Hydra* tending to confirm the model are reported. Possible detailed realizations of the model in terms of, biochemical control circuits within the cell, are conjectured and discussed to show that the formal features of the model can be realized by well-recognized biochemical processes.

1. Introduction

A basic problem arising in the study of embryonic development is how the global or field aspects of the developmental process arise from the known properties of single cells and their interactions. While it is true that globally-orientated disciplines such as analytical topology can provide a rigorous logical framework within which to contain any specific theory of development, such a generalized context can provide answers to rather general questions only. An example of this approach is provided by Rene Thom's (1969) interesting demonstration of the categories of instability that can arise in the four-dimensional space-time of the developing embryo as a result of biochemical processes taking place in multi-dimensional concentration space. This kind of analysis, which proceeds without requiring the existence of cells in the embryo, may be relevant to an understanding of certain embryological processes such as invagination and neurulation. However, it does not contribute any insights into the specific processes which originate at the cellular level in the embryo and result in the emergence of tissues and organs as ordered aggregates of differentiated cells. It is the cellular level which is the relevant one for this particular problem, as is attested by such simple observations as the absence of cells which are half nerve, half muscle, or any other combination of differentiated states. The single cell is the structural unit of the differentiated tissue. A tissue is a spatially ordered set of such units, with a particular pattern of interaction among them. The challenge of embryology to molecular and cell biology is to account for the way in which such spatially organized structures come into being as a result of interactions which arise from and are controlled by reasonably well-understood cellular processes.

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sequence of events in which spatial and *temporal* organization in the developing tissue mass are intimately connected. Before any visible sign of spatial organization is evident in a tissue, it is neceessary that there be a specific distribution of differing cell states.† This specific spatial distribution of states, developing with time, must result from some kind of interaction or communication process among the cells in the tissue. Here we examine how this space-time connection can be realized in terms of the physiology of single cells and their interactions.

Most theories of pattern formation lean heavily upon the idea of a gradient of chemical substance or of metabolic activity as the basic space-structuring factor in the developing embryo. Positional information would then be carried by such a gradient, cells behaving in particular ways according to the value of a concentration or an activity in particular parts of the embryo or tissue. As cells differentiate in directions determined by these different values of the concentration or activity, they produce new substances and alter their metabolism. New gradients are thus generated so that the process continues until the system comes to some equilibrium or quasi-equilibrium in the final differentiated state.

A critique of the major theories based upon the gradient concept has been presented by Webster (1964), who pointed out some of the difficulties such theories encountered when faced with the phenomena of regulation and regeneration. Webster (1966) has proposed a gradient theory to account for those phenomena. By replacing the gradient of chemical substance or metabolic activity by one of threshold values for a particular type of response to a particular inducer, he has constructed an elegant scheme with simple and reliable regulation, achieving size independence of pattern formation in simple linear systems. Such a scheme fails, however, when one attempts to account for periodicities in embryonic structures, such as repeated somites, body segments, digits, periodic pigmentation patterns, etc. Gradient-based theories must then have recourse either to Turing-type standing waves of chemical substances or morphogens (Turing, 1952; Maynard Smith, 1958), or postulate the prior existence of a periodicity in the spatial distribution of threshold values. The latter evidently does not account for the origin of the periodicity, and is not advanced as an explanation. The problem simply gets transposed to a different level, that of accounting for threshold distributions. Turing-type periodic waves remain a possibility, but the problem of regulation for such a model is severe. So also are the problems of the time required for the standing wave to be established and its stability in the presence of cell movement. The absence of any clear, simple, unifying principles emerging from substance gradient theories encourages one to

† We have in mind a definition of cell state like that employed by Goodwin (1969).

consider the possibility of a radically different approach to the whole question of pattern formation and morphogenesis in embryonic systems.

One such approach was inspired by a formal statement of the linear pattern-formation problem by Wolpert (1968), who considered possible solutions to the "French Flag problem", that of dividing a system into three regions whose linear ratios remained invariant to size changes in the whole. The solutions given by Apter (1966) and by Wolpert (1968) have the characteristics of algorithmic procedures for computing the positional information that is to be assigned to each cell in the aggregate. They are representative of a whole class of solutions which are based upon digitalized computing procedures. Each cell is regarded as an automaton which can perform certain digital operations such as counting, telling left from right, passing specific information to neighbors according to what information they receive, etc. Although these may seem to be highly unlikely activities for cells, there are biochemical processes which apparently function in near-digital form. An example is the highly discontinuous behavior of the enzymes deoxyribonuclease and thymidine kinase in the differentiating spore cells of lilly anthers (Hotta & Stern, 1963). One particular digital concept, that of *threshold*, has been in the embryological literature for many years, necessitated by the primitive observation referred to above that differentiation at the cellular level is an all-or-none process. Such either-or responses of cells form an essential part of all gradient theories of pattern formation, and the further elaboration of digitalized processes in cells, such as counting, is simply an extension of the threshold concept. It hardly needs to be emphasized, however, that such a process must ultimately be interpreted in terms of biochemical or physiological mechanisms of some kind. The absence of explicit interpretations of most automata-based models, and hence their lack of predictive power, remains a deficiency.

A general analysis of the problem presented by pattern formation in embryos and the essential concepts required for its resolution has been presented by Wolpert (1969). Basic to his exposition is the concept of positional information and the assumption that whatever physiological processes are involved in generating this information, they are likely to be universal throughout the animal, and possibly also the plant, kingdoms. Wolpert's analysis demonstrates that in regulation embryos, such as the sea urchin or the amphibian, the establishment of an embryonic axis in relation to which cells differentiate according to their position requires (1) a reference point specifying the origin of the axis, the point from which measurement begins, (2) a direction for measurement along this axis, and (3) a scale-adjustment mechanism which can alter the units of measurement along the axis so that the total number of units on this axis remains invariant

to changes of absolute axial length. In Wolpert's terminology these are (1) a reference cell or region, termed α_0, (2) polarity, which is the direction in which positional information is measured, and (3) in bipolar systems a second reference cell or region, termed α_0', at the opposite end of the axis from α_0, which in some manner adjusts the size of the measurement units to fit the available axial length. Given such an axis, a cell located at some point along the axis will have available to it information which is specific for that position, and can then differentiate according to some 'code' for interpreting this positional information. The distinction between positional information in relation to an embryonic axis and the interpretation of this information is a very important one, for it makes the assumption of universality of the nature of positional information highly plausible. Nature being conservative, it seems likely that the method of establishing reference axes in embryos is basically the same throughout the phyla. Different species would then employ different codes for interpreting the positional information provided by these axes, thus achieving variety of phenotype while conserving a basic epigenetic process. Some of the many consequences of this original and important idea are explored by Wolpert in his paper.

Wolpert deliberately avoids any attempt to specify the detailed physiological or biochemical nature of the processes involved in the establishment of co-ordinate axes in embryos. By conducting his argument in terms of formal constructs such as the α_0 cell or region and polarity, he can explore the general consequences of his analysis without making unnecessary assumptions about cellular mechanisms. This leads to some very interesting interpretations of a number of observations in experimental embryology. It was Wolpert's formal analysis of the problem of pattern formation and his appeal for a new approach which first inspired the model which we will present in this paper.

We imposed upon ourselves the constraint of utilizing only plausible, in fact familiar, biochemical and physiological properties of cells in constructing the model. The property we have exploited which gives this model its novelty is the relatively recent demonstration that cells are temporally organized systems, this temporal organization arising from oscillatory behavior in the cell's physiological control processes (Pittendrigh, 1961). Whatever its behavior, whether cleaving, growing and dividing, 'resting', or differentiating, there is a well-defined sequence of biochemical and physiological processes which defines the behavior of a cell. Furthermore, periodic or rhythmic activity is the most commonly encountered mode of cell behavior. The cycle of cell division is for all cells a basic dynamic mode of organization insofar as any existent cell must at one stage have undergone this cycle and have been organized in this periodic mode. Cell or nuclear

division is, furthermore, the initial process which cells undergo in nearly all embryos. It seems natural to suppose that the temporal organization present in the earliest embryo should form the dynamical basis for the future development of the whole embryo. The question is then how this temporal order can be used to generate spatial organization throughout the developing cell mass. Certain aspects of this problem have been explored by Waddington (1965) and by Goodwin (1965, 1967).

In the present paper this problem is resolved by postulating certain initial conditions in an embryo together with interactions between cells arising from the transmission of charged ions or small molecules from cell to cell by some kind of functional coupling. In this way periodicities in one cell can affect those in its neighbors. A particular pattern of interactions can result in an ordering of the phases or relative timing of biochemical periodicities in a cell as a function of its position in the whole aggregate. Positional information is than present in the form of a specific temporal pattern of biochemical periodicities in a cell occupying a particular position in the aggregate. All cells may be making exactly the same substances initially, but the temporal order in which these substances are made will vary in an ordered manner along spatial axes, with the consequence that the potentialities of cells for the synthesis of specific substances will vary with position in the aggregate. The cells will then differentiate in accordance with these biosynthetic potentialities, and ordered heterogeneity will appear in the tissue. The differentiation of the cells will in turn affect their periodicities, thus resulting in altered interactions between the cells and new patterns of positional information, again present as phase-differences of periodic events in cells as a function of their spatial locations.

The spatial and the temporal aspects of embryonic development thus become very intimately, indeed indissolubly, connected, and it becomes a truism to say that the developmental process is one taking place in four dimensions. Time, in the form of ordered periodicities, is an essential ingredient. Aperiodic and periodic patterns in one or more dimensions, size-sensing and regulation, can all be produced quite simply by slight extensions of the basic time-space ordering mechanism, as will be discussed in detail. The model thus has universal features. It also has predictive power: the novel postulate of periodicities as a basic space-organizing element in coupled cells leads to clearly-defined procedures for observation and experimental interference. The cellular periodicities function as local developmental clocks which should be both detectable, and manipulable by the introduction of appropriate periodic stimuli from the environment. The model is thus open to investigation by means well within current technological capabilities.

Another feature of the model is its high informational capacity, making it applicable in principle to those aspects of embryonic development which demand a high degree of specificity such as neurogenesis, muscular innervation and immunopoiesis. The existence of tight junctions or similar means of intercellular communication in the adult organism suggests that the mechanisms proposed here may have relevance to postembryonic developmental phenomena such as learning and oncogenesis.

2. Spatio-temporal Ordering of Coupled Cells

To summarize the arguments of the Introduction, embryological evidence requires the ordering in space and in time of developing tissues, regarded as collections of coupled cells, but it does not indicate any particular mechanism whereby this ordering is effected. In the present section we give the general considerations underlying a model of such an ordering mechanism to be constructed and elaborated in subsequent sections.

We base our entire theoretical structure on the following three propositions:

(I) Physiological processes within individual cells are temporally organized.

(II) Intercellular coupling causes the temporal organizations of the different cells in a tissue to vary in a well-defined manner with their positions in the tissue.

(III) This spatial organization of temporally-ordered cells can provide the positional information necessary for developmental processes.

These propositions define the first of four different levels at which the problem of constructing the ordering mechanism can be explored; they formulate an abstract and universal conceptual model. Universality may well hold throughout the lower levels of abstraction. At the next level, patterns of biochemical events can be postulated which together represent a concrete, formal realization of the abstract model. The individual biochemical events can be specified in terms of explicit biochemical control circuits at the third level. Finally, at the fourth level, particular molecular species can be identified with the roles defined at the third level. Attention is focused on the first level in the present section and on the second level in the next several sections. The highly speculative discussions carried out later at the third and fourth levels are intended only to demonstrate that concrete realizations of the model are possible and to elicit common features of various representations which are susceptible to experimental test. The paucity of experimental information bearing directly on the questions we raise and the complexity of the associated problems make it unlikely that much detail can survive as correct even at the second level of abstraction.

Our work should therefore be regarded only as exploratory, an endeavor to start the subject blocked out by our three propositions.

The evidence for the first proposition is twofold. On the one hand, the fact that cells are temporally organized follows immediately from the existence of the cell division cycle (Goodwin, 1969). The occurrence of physiological periodicities in single cells with frequencies different from that of cell division is well known from studies of circadian rhythms and biological clocks (Hastings, Astrachan & Sweeney, 1961; Bruce, 1965; Strumwasser, 1965). High frequency oscillations have been observed *inter alia* in the respiration of yeast cells (Betz & Chance, 1965) and in genetic transcription (Imamoto, 1968; Baker & Yanofsky, 1968). On the other hand, the existence of developmental clocks has been demonstrated for such organisms as *Drosophila* (Pittendrigh, 1965) and *Pectinophora* (Minis, 1965).

Recent investigations of electrical communication between embryonic cells such as those of Potter, Furschpan & Lennox (1966) are of great relevance to proposition II. They showed that there is electrical communication throughout the whole of the early embryo but that this is lost between the embryo and the yolk sac at later stages. This communication is inferred to arise from easy transport of small ions between neighboring cells via tight junctions, as observed with electron micrography by Peachey & Rasmussen (1961). Proposition II does not, however, require that the coupling be specifically electrical (i.e. ionic) in nature; any molecular species could act as the intercellular signal. Nor do we wish to imply that tight junctions are necessary for intercellular coupling. Signalling between cells separated by millipore filters could still exist, though attenuated (Saunders & Gasseling, 1963).

Proposition III is nearly self-evident. If cells are temporally organized in ways that differ according to their positions within an embryonic tissue, they are already predifferentiated according to position. Suppose, for example, that differentiation is initiated by threshold concentrations of different inducer molecules. Suppose also that the inducer which is produced and its rate of production depend on the temperal organization of the cell. Then different inducer molecules are produced at different rates in differing parts of the embryo according to a well-ordered pattern. As the concentration of each inducer reaches its threshold, differentiation is initiated according to that same pattern.

3. A Time Base for Development

We now postulate a pattern of biochemical events which leads to synchronization of the cells in a developing tissue, and thence to a local time base for development. Suppose there to occur within each cell an auto-

nomously periodic event *S* involving a number of different biochemical species. (The possible biochemical nature of this event is considered in section 6.) One consequence of this event is the generation of a brief signal which can be transmitted via functional coupling to nearest neighbor cells. The signal, which we can imagine to be either small ions or small molecules, induces event *S* in the neighboring cells, which in turn induce *S* in their neighbors. A wave of occurrence of *S*, the *S*-wave, propagates outward from the cell or region of cells of highest autonomous frequency, the pacemaker or dominant cell or region. We can guarantee forward propagation of the wave by supposing there to be a refractory period following the signalling aspect of the *S*-event during which *S* cannot be induced. A cell is receptive or sensitive when it is not in the refractory period. The wave then recurs regularly and stably at the pacemaker frequency.

The process described above is simply the entrainment of a set of coupled non-linear oscillators by the fastest oscillator among them, a problem considered in detail by Winfree (1967). An example of stable synchrony of oscillatory activity in a population of cells is provided by the studies of Sweeney & Hastings (1958) and Hastings *et al.* (1961) on the marine dinoflagellate *Gonyaulax polyedra*. Populations of these unicellular organisms maintained under constant environmental conditions showed a stable oscillation of bioluminescence, respiratory activity and photo-synthetic capacity with a mean period of about 24 hours at 21·5°C. There was also a population rhythm of cell division with the same period, although the mean generation time of the cells was 36 hours. Thus the periods of the physiological oscillations were shorter than the mean periods between divisions of individual cells. The population rhythms were stable indefinitely once they had been established by an initial entraining environmental signal, but they do not, apparently, arise spontaneously in an initially asynchronous population. This implies that the interactions between cells in such populations are relatively weak. We are postulating that the interactions between cells in an embryonic tissue are sufficiently strong to result in the establishment of a single frequency of the periodic *S*-event throughout all the cells of the tissue, as occurs, for example, in the cells of the lamprey heart.

We suppose the time for wave propagation across an entire tissue to be short on the time scale of developmental processes. The cells are then synchronized in relation to event *S*. The symbol *S* thus stands for the synchronizing property of the event. The *S*-event provides a local clock for the development of the tissue.

If the signal is purely electrical in character, its time of transmission across highly permeable membranes could be in the millisecond range. The rate limiting step in the propagation of the *S*-wave would then be the delay

Δt_s in the production of an S-signal by a cell after receiving one. The value of Δt_s could be of order one second or less. Propagation of an S-wave across an embryonic tissue 200 cells in linear size could therefore require of order three minutes or less. This is short on the time scale of development, i.e. several hours to a day or more. For the event S to provide an accurate developmental clock, there must be many ticks of that clock between major developmental events. The period of S must be at least an order of magnitude shorter than characteristic developmental times. We therefore imagine T to range from about one minute to one hour depending on the organism and the stage of development. As will appear more clearly later, T must also be long relative to characteristic biochemical generation and decay times to allow for the generation of complexity and specificity.

4. Positional Information

(A) FREQUENCY GRADIENT

If the distribution of autonomous S-frequencies were random throughout the tissues, so would be the position of the pacemaker region. A randomly placed pacemaker would be of little value for establishing positional information. We postulate, instead, a gradient of autonomous frequencies within the tissue such that the maximum frequency and hence the pacemaker region occurs in a well-defined position. The origin of this frequency gradient in the early embryo could be the gradient of nutritive (yolk) and other materials within the egg, which is preserved by cell division. The cell division frequency and metabolic rate per cell vary inversely as the yolk gradient. A direct correlation among metabolic activity, cell-division rate, and the S-frequency would be natural. We would, therefore, frequently expect to find the pacemaker region of a relatively undifferentiated embryo at the pole of an axis of developmental symmetry, where the cells are small as a consequence of rapid division. In the later stages of development, the frequency gradients in tissues would arise during their individuation.

The propagation of S-waves away from a spatially localized pacemaker region establishes a direction in an embryonic tissue which may be identified with the concept of polarity discussed by Wolpert (1969). This polarity is a property of the whole tissue when synchronized by a pacemaker; it is not a property of single cells. It establishes an embryonic axis in relation to which positional information may be measured. The nature of this information and how cells respond to it will be considered in section 4(B). The frequency gradient can be of two characteristic types as displayed schematically in Fig. 1(a) and (b). In Fig. 1(a), the S-frequency falls rapidly for a short distance and then remains nearly constant, the initial gradient merely

being sufficient to establish the pacemaker region. In Fig. 1(b), the *S*-frequency continues to fall throughout the tissue. Other frequency variations are of course admissible as long as the requirement of a well-defined maximum is satisfied.

It is well known that coupled non-linear oscillators can be entrained only over a finite range of unperturbed frequencies. We therefore expect that when the frequency gradient becomes too large, entrainment can occur only over a limited number of cells. The tissue will then tend to break up into regions over which the range of unperturbed frequency is just that for which entrainment can occur. Within such regions the pacemaker cells will

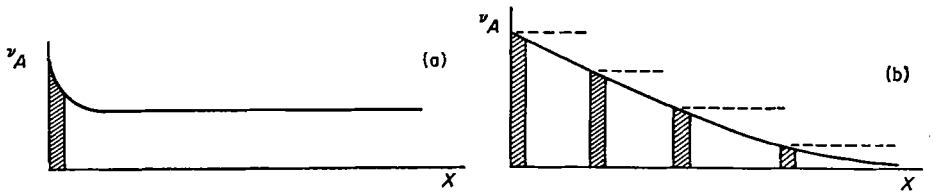

FIG. 1. Two possible frequency gradients. The shaded region corresponds to the pacemaker cells.

(a) A rapid initial fall sufficient to establish a dominant region followed by no further significant frequency change.

(b) A continued frequency decrease which, if large enough, can give rise to breakdown of entrainment, multiple pacemakers, and frequency steps in the *S*-wave frequencies of the several entrained regions, as indicated by the dotted lines and the correlated shaded regions.

again be those with highest unperturbed frequency. However, cells on one side of a boundary between two separately entrained regions will receive signals of the wrong frequency from cells on the other side of the boundary. This would interfere with the synchronization process in each region. By assuming that the refractory period of the *S*-event is a substantial fraction of its period, we can both minimize the above interference effect and establish a simple and reliable model for entrainment over a well-defined frequency range.

Let the period of the *S*-event be T and the duration of the refractory period be $r_s T$ ($r_s < 1$). For simplicity we ignore the duration of the signalling part of the *S*-event relative to $r_s T$ and $(1-r_s)T$. A given cell can be triggered only after the refractory period following the previous *S*-event is over. The shortest period with which a given cell can be driven is therefore $r_s T$, its refractory period. We suppose r_s not to vary within the tissue, even though T does. The ratio of the minimum autonomous frequency within an entrained region (that of the last cell) to the maximum autonomous frequency (that

of the first cell) is then r_s. The ratio of the frequency of the $(i+1)$th region to that of the ith is similarly r_s, i.e.

$$\nu_{i+1} = r_s \nu_i$$

and

$$\frac{\Delta \nu_i}{\nu_i} = \frac{\nu_i - \nu_{i+1}}{\nu_i} = 1 - r_s.$$

If ν_{max} is the maximum frequency in the tissue, the frequency of the ith region is $r^{i-1}\nu_{max}$. The frequency of the last region is consequently given by

$$\nu_\eta = r_s^{\eta-1}\nu_{max}, \tag{1}$$

where η is the number of separately entrained regions. The minimum autonomous frequency in the tissue, ν_{min}, must satisfy

$$\nu_{min} \geqslant r_s \nu_\eta, \tag{2}$$

otherwise there would be more than η regions. Putting (1) and (2) together gives the relation

$$\nu_{min} \geqslant r_s^\eta \nu_{max}, \tag{3}$$

from which we derive an expression for η,

$$\frac{\ln \nu_{min}/\nu_{max}}{\ln r_s} - 1 < \eta \leqslant \frac{\ln \nu_{min}/\nu_{max}}{\ln r_s}. \tag{4}$$

From (4) we see that for a given frequency gradient there is a complementarity between the number η of separately entrained regions and the degree of interference between them. When r is small, there will be few regions and much interference between them; whereas as $r \to 1$, the number of regions becomes large and the interference small. It should be remembered that in tissues which do not show frequency steps, the refractory period need not be so long.

In summary, the S-wave propagates from the dominant region of each entrained portion of the embryo to its boundaries; each entrained portion is virtually autonomous; and the S-wave frequency shows the step-like dependence of position depicted by the dotted line of Fig. 1(b). The size of the ith frequency step, $\nu_i - \nu_{i+1}$ is $(1-r_s)\nu_i$, where ν_i is the dominant frequency of the ith region, counting from the highest frequency region as the first.

If the processes of differentiation are independent of the S-frequency, the frequency step pattern carries no positional information. If, on the other hand, the contrary is true and differentiation depends on or is correlated with the S-wave frequency, the frequency-step pattern of Fig. 1(b) already provides information for regional tissue differentiation. It is clearly not adequate for specification of fine detail in pattern formation.

(B) PHASE GRADIENT

In order to obtain finely detailed positional information, we require a second periodic biochemical event, P, in each cell whose time of occurrence after the S-event depends in an orderly way on the cell position relative to the dominant region. Here P stands for positional or phase information. Let us suppose that in a free-running or pacemaker cell, P would be caused by S, hence would have the periodicity of S, and would occur at a definite phase ϕ_0 after S.

Thus P is not an autonomous event; it cannot occur independently. In a cell which is being driven by the pacemaker region and thus has a frequency of S which is greater than its natural or unperturbed frequency, we suppose that the S-event is sufficiently altered that P is no longer caused by this driven S-event, which we therefore call S_d. P is instead initiated by a signal associated with the P-event in a neighboring cell. If the time taken for the signalling and induction of P is appreciable, the P-event in a cell will be significantly phase shifted relative to the P-event in the neighboring cell inducing it. Further, if the cells are refractory to P after P occurs, there will be a periodic P-wave propagating outward from the dominant region more slowly than the S-wave. The phase of occurrence of P relative to S_d in each cell will increase monotonically with the distance of that cell from the dominant region. Figure 2 shows how cell state depends on cell position in our model.

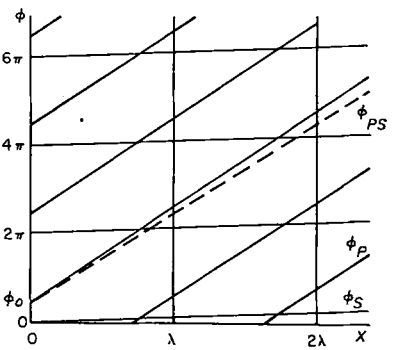

FIG. 2. Phase of occurrence ϕ of S_d and P events as a function of cell position x. The dominant region is indicated by a heavy line segment. Note that the phase of P, ϕ_P increases more rapidly with distance from the dominant region than that of S_d, ϕ_S. The phase difference between them, ϕ_{PS}, thus increases correspondingly. If there is no mechanism limiting the increase of ϕ_{PS} with distance, the cell state as measured by ϕ_{PS} is periodic with period λ. If ϕ_{PS} is restricted to a maximum value ϕ_M, the system is size independent with some repeat for $\phi_M - \phi_0 > 2\pi$ and no repeat for $\phi_M - \phi_0 < 2\pi$ as disucssed in the text.

For the above model to be consistent with proposition III, the phase gradient must constitute positional information which can be used reliably for development. Suppose that a particular range of the phase difference ϕ_{PS} between successive S_d- and P-events within a given cell is required for the production in appreciable amounts of a particular inducer, I, as shown in Fig. 3. The inducer can therefore be made only at a location in the embryo corresponding to that range of phase differences. A fixed amount ΔI of I will be made each period in cells at that location. If the decay in I per cycle is small compared to ΔI, it will accumulate and exceed its threshold value I_0

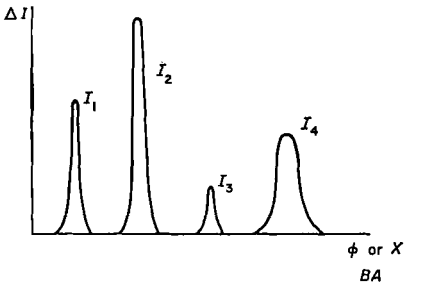

FIG. 3. The number ΔI made per period of several different inducer molecules as a function of phase difference ϕ_{PS} beween S_d- and P-events or, equivalently, of position x along the embryo. Other agents as well as inducers can, of course, be made by the same mechanism.

in a number of periods equal to the first integer exceeding $I_0/\Delta I$. There is a concentration range centered at I_0 within which the cell state changes from no induction to induction, which we call the transition range of I. If ΔI is larger than this transition range, a particular differentiation is reliably initiated at a particular time and at a particular place in the embryo. The inductive mechanism functions as a counter, $I/\Delta I$ being the number of cycles or periods counted.

If the range in phase over which a developmental agent such as I is made is fairly small, there will be space along the embryo for the manufacture of a number of distinct agents in distinct positions (cf. Fig. 3), all reaching threshold at characteristic times. The phase gradient mechanism outlined above is therefore a rich source of the kind of positional information needed in development, the richness arising from the use of temporal organization as the information. Moreover, the positional information emerges in a time-ordered fashion.

(C) INITIATION OF THE PHASE GRADIENT

The advantage of a long refractory period, $r_s \sim 1$, for reliability and stability has been considered in (A) of this section. However, since a long refractory period results in a short period of sensitivity, cells can only communicate within a narrow range of relative phases. If the relative phases of the S-events in the cells of a developing aggregate were to be randomly distributed at some initial time t_0, then the time required for synchronization could be very long and might not, in fact, ever be completely realized. In the latter case, the pattern of positional information would be fragmented. We conjecture that in an actual embryo such an initial condition never occurs. The early cleavages are synchronous, but this synchrony begins to decay after the fourth division or later. This initial synchrony of cell division we take to indicate an initial synchrony in all of the biochemical activity of the cells. If the S-event commences when the cells are in synchrony or shortly thereafter, then the difficulty of a random distribution of initial phases is avoided. Providing that the cell division cycle does not interfere with the S-event, i.e. the state of a cell as regards the S-event is inherited by its daughters, orderly propagation of S-waves will occur and similarly for P-waves, and positional information can be established via the phase gradient.

Disaggregation and random mixing of embryonic cells, followed by a reaggregation, would result in a random distribution of relative phases. In consequence of this and the assumed long refractory period, such a reaggregate might never achieve a state of coherent S-wave propagation, and thus would have fragmented positional information and show incompletely organized development. This could account for the observations of Townes & Holtfreter (1951) on the aberrant and incomplete differentiation of embryonic reaggregates.

(D) PERIODIC AND APERIODIC PATTERNS; SIZE INDEPENDENCE; REGULATION

A periodic pattern is the natural consequence of the phase shift mechanism which we have thus far constructed. The spatial period is $\lambda = (2\pi/\Delta\phi_{PS})d$, where $\Delta\phi_{PS}$ is the mean change in relative phase of P and S in moving from one cell to its neighbor and d is a mean cell diameter. The periodicity does not show up until the length L exceeds λ, or equivalently until the total number of cells N along the direction in which the S- and P-waves propagate exceeds $2\pi/\Delta\phi_{PS}$. The number of periods increases as N increases.

Aperiodic patterns can occur in embryonic tissues for which N is less than $2\pi/\Delta\phi_{PS}$. However, these patterns would change as N is changed in

any way; that is, such embryos would be non-regulative, with pattern dependent upon size. In order to achieve regulation in an aperiodic system, the phase difference ϕ_{PS} cannot depend solely upon cell position x, or cell number $n = x/d$, alone but must depend instead on the relative cell position x/L. A mechanism which gives such a size-independent phase pattern must (1) limit the phase difference ϕ_{PS} to some maximum value $\phi_M < 2\pi + \phi_0$ and (2) set $N\Delta\phi_{PS}$ equal to $\phi_M - \phi_0$ on the average. The latter requires the reduction of $\Delta\phi_{PS}$ with increasing N.

Consider a tissue with $n\Delta\phi_{PS}$ larger than ϕ_M, where $\Delta\phi_{PS}$ is the phase shift that obtains when the S- and P-waves propagate in the absence of a regulatory mechanism. ϕ_{PS} will reach ϕ_M at some cell (or set of cells) with $n = (\phi_M - \phi_0)/\Delta\phi_{PS} < N$ cells away from the pacemaker region. Suppose that whenever ϕ_{PS} reaches ϕ_M a third event R (for regulatory or reset) is initiated which, by signalling, induction, and refractory processes like those already invoked for S and P, propagates outward in all possible directions from the nth cell. During R a slowly decaying molecular species Q is produced, which has the effect of reducing the phase shift $\Delta\phi_{PS}$ and possibly ϕ_0 as well. During the next P-wave ϕ_{PS} will reach ϕ_M at larger n, and the R-wave is repeated, until finally, ϕ_{PS} remains under ϕ_M throughout the tissue. As Q slowly decays in each cell, ϕ_{PS} will reach ϕ_M at the end of the tissue opposite the dominant region, and an R-wave will recur, replenishing Q. This regulatory process is stable.

If such a system is reduced in size, the maximum phase shift is reduced below ϕ_M. The concentration of Q will gradually decrease, increasing the maximum phase change towards ϕ_M. When ϕ_M is reached the R-wave regulation sets in again. The pattern is, therefore, size independent (cf. Wolpert, 1969).

Periodic patterns can be made size independent by a slight modification of the above R-wave scheme. Suppose that the production of Q is a threshold process and that the threshold is reached only after m successive R-events have taken place within a single period, where m is an integer. This requires m regions where $\phi_{PS} = \phi_M$ modulo 2π along the embryo, corresponding to a total phase shift of $\phi_M + 2(m-1)\pi - \phi_0$. The resulting pattern is m-fold periodic, stable, and size independent.

We expect the regulatory mechanism to operate only within a certain range of N. The lower bound is set by the requirement that $n\,\Delta\phi_{PS}$ be greater than $\phi_M - \phi_0$ when $\Delta\phi_{PS}$ is the unregulated phase shift. The upper bound is set by the requirement that P continue to propagate. Clearly too much compression of P in time can interfere with its propagation mechanism. In other words, there will be a critical concentration of Q above which propagation of the P-wave cannot be triggered.

This size-regulated phase-gradient scheme can be combined with the frequency-step scheme discussed in section (A) to provide exceedingly detailed positional information if we suppose the developmental processes to be sensitive to frequency as well as to phase. The frequency steps provide a coarse position scale, and the phase gradient superposes a fine scale which is independent of the size of the individual regions of constant frequency. Within a region of constant frequency, the phase change may be less than or greater than 2π, i.e. there may or may not be periods. Similarly, there may or may not be regulation. The overall pattern, as shown in Fig. 4

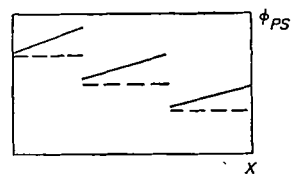

FIG. 4. Combined frequency step and phase gradient scheme. The frequencies ν of the *S*- and *P*-waves in the different regions of entrainment are shown as dotted lines. The PS_d phase difference ϕ_{PS} runs from ϕ_0 to ϕ_1 as position co-ordinate x increases within each region because of the *R*-wave size regulation.

can have a limited size independence even in the presence of the frequency steps. Size independence of the frequency-step pattern requires size independence of the underlying pattern of autonomous frequencies according to the discussion of section A. Provided the frequency of the *S*-event in a given cell is inherited by its daughter after cell division, the autonomous frequency distribution, and therefore the pattern of frequency steps, is invariant with respect to size changes caused by cell division and growth. Invariance with respect to size changes caused by section or fusion would need an additional regulation process, but seems an improbable requirement in the circumstances in which such finely detailed positional information as is provided by the combined frequency-step, phase gradient scheme is required. As an alternative to this scheme, more than one kind of *P*-wave could emerge from the pacemaker to set up finely detailed information.

It should be realized clearly that the considerations of section (B) and the present section have led us to a model which regulates in time as well as in space. Those aspects of cell state which are important for development depend primarily on a phase difference ϕ_{PS} which is a time difference t_{PS} measured relative to a period T,

$$\phi_{PS} = 2\pi t_{PS}/T. \tag{5}$$

Changes in the environment which slow down or speed up embryonic

cellular activity, and in particular change T, have no effect on ϕ_{PS} because t_{PS} changes in proportion with T. Thus, the time ordering of developmental events is stable relative to such environmental perturbations even though the time of development may be affected. Size regulation occurs in our model because the phase difference ϕ_{PS} depends only on relative position or cell number,

$$\phi_{PS} = \phi\left(\frac{x}{L}\right) = \phi\left(\frac{n}{N}\right), \tag{6}$$

where x is cell position, L is tissue length, n is the corresponding cell number, and N is the total number of cells along L. To summarize, the time and space dependence of functions f of cell state is given by

$$f = f\left(\frac{t}{T}, \frac{x}{L}\right) \tag{7}$$

in our model.

(E) SIZE SENSING

The essential feature of the size-regulation mechanism of the previous subsection is the triggering of the R-wave when ϕ_{PS} reaches a critical value ϕ_M in a cell or set of cells. The triggering of a propagating wave when a certain phase difference is first reached within a tissue provides a critical size sensing mechanism together with a basis for an active tissue-wide response to the critical size as well as subsequent regulation of the response, the critical size being $N_c = (\phi_M - \phi_0)/\Delta\phi_{PS}$. The mechanism described here for size independence of positional information is therefore of still wider functional utility. The onset of pacemaker activity in developing heart tissue when a certain size is reached may be an example of its occurrence. For size sensing in a periodic system, a mechanism like that proposed for the regulation of periodic patterns can be used.

A weaker size sensing mechanism is already implicit in our description of the way the phase gradient is used for positional information in the production of inducer molecules. There need be no propagating event to ensure tissue-wide response to the critical size. An inducer or repressor could then be produced at the end of the tissue away from the pacemaker region when the critical size is reached. A repressor so produced could, for example, then diffuse and stop cell division, the cell division process being slow enough for diffusion control to work.

(F) ORGANIZING WAVE PROPAGATION IN TISSUES

We have thus far introduced three waves, the S-wave for synchronization, the P-wave for positional information, and the R-wave for size regulation.

Together they form a set of *organizing* waves (or *O*-waves) which organize development in the model we are constructing. The relation between these *O*-waves and the action of the amphibian organizer will be considered in detail in section 7.

A fundamental embryological question is what, in general terms, is the interrelation between the geometry of an embryo or of an embryonic tissue and the pattern of positional information within it? Our model yields a clear and simple answer. The pattern of positional information is the pattern of the surfaces of constant phase difference propagating outward from the pacemaker or dominant region. The geometry of the embryo enters through the boundary conditions to which the waves are subject, which in turn follow from the existence of a refractory period, as discussed in the Appendix. In the limit of a many-celled tissue, the discrete cellular structure may be ignored, and the waves will propagate through the embryo as through a continuum with a propagation velocity dependent upon local cell size. Very general mathematical considerations can then be brought to bear on the problem of the relation between the qualitative description of the pattern of positional information and of the embryonic geometry simply because the former derives from the solution of a partial differential equation governing the wave propagation subject to the boundary conditions. The derivation of the wave equation and the boundary conditions in the continuum limit appropriate to a many-celled tissue is given in the Appendix.

5. Higher Dimensional Ordering

The spatial ordering process introduced in section 4(B) and discussed analytically in the Appendix provides positional information in the form of an ordered one-dimensional sequence of surfaces of constant phase difference ϕ_{PS} propagating outward from a single pacemaker region which defines a pole. The longest trajectory (locus of normals to the wave fronts) present in the tissue can be taken as the axis of development along which a one-dimensional position can be measured within the embryo. The co-ordinate x used in section 4 (see Figs 1 to 4) should be so interpreted.

Figure 5 shows the dominant region (taken as a point for simplicity), the axis of development, and the surfaces of constant ϕ_{PS} for some typical embryonic geometries. The figures are plane sections through hypothetical three-dimensional embryos, illustrating the one-dimensional character of the positional information and its relation to the embryonic geometry.

We note that the developmental axis originates in the pacemaker region and terminates at the point where the phase has its maximum value. This will be the sole point of origin of the *R*-waves in an aperiodic system; we may call it the regulator or regulating pole. The developmental axis then

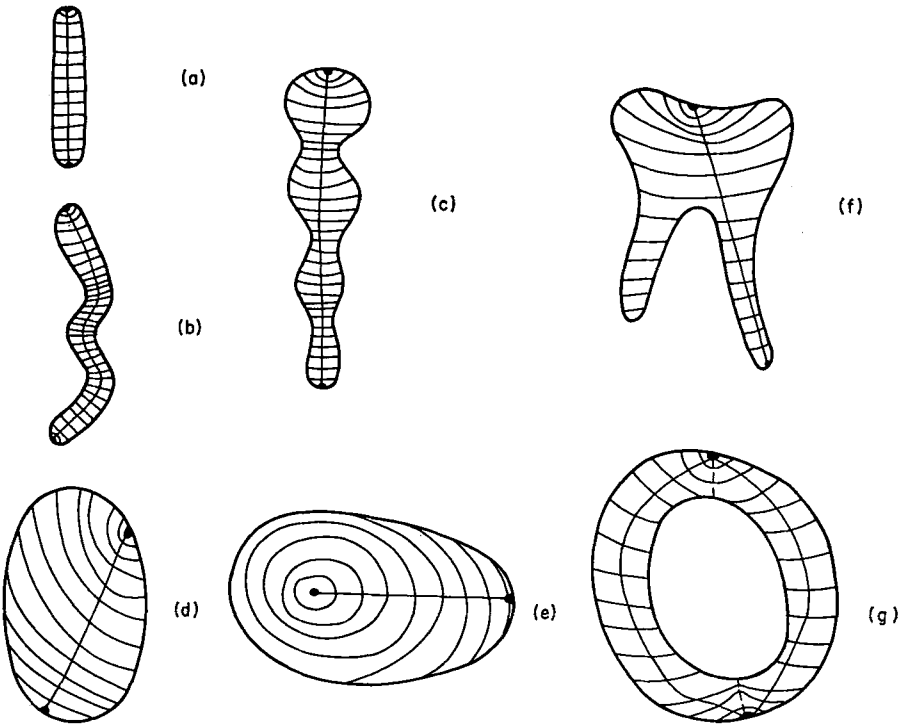

FIG. 5. Plane sections through hypothetical embryos having typical embryonic geometries. The dominant region is represented by a closed circle, the presumptive regulating region (which need not be present) by an open circle, and intersections of the plane sections with surfaces of constant phase difference ϕ_{PS} are shown as families of curves. Axes of development, as defined in the text, are also shown running from dominant to regulating regions. The boundary conditions on the P and S waves cause detailed changes of shape in the surfaces of constant phase near the boundaries of the embryo which are not accurately drawn. Each figure may just as well be regarded as representing a discrete tissue within an embryo as an entire embryo. (a) The embryo is cylindrically symmetric, and the developmental axis coincides with the axis of symmetry. (b) and (c) are topologically equivalent to (a). Embryo (c) corresponds to the size-dependent periodic case of section 4. Although (d) shows the same external figure as a polate spheroid, the internal cell arrangement is less symmetric. The dominant region is off, and the developmental axis distinct from, the axis of symmetry. (e) Shows the contours of constant phase in a single plane sheet of cells or in a flattish embryo made up of stacked sheets. Complex forms topologically equivalent to (e), such as a hollow version of example (c), can be achieved by distortion of (e). Because the surface of embryo (f) is reentrant, the wave fronts become doubly connected sections, as is also the case for (g). One developmental axis still suffices, however.

runs from dominant region to regulating region. In terms of Wolpert's (1969) terminology, the pacemaker region is α_0 and the regulating pole is α'_0. In a system with multiple periodicities, there will be several regulating regions.

How can we establish two and three-dimensional ordering within a developing tissue? The simplest solution is to have one or two secondary centers which act as origins for the propagation of new *P*-waves, giving rise to two or three sets of intersecting surfaces of constant phase difference. As long as there is no interaction between the *S*-event and secondary *P*-events, there is no need to have each center send out its own characteristic *S*- and *P*-waves. One *S* wave from the original dominant region suffices to establish a time base throughout the entire tissue. At some later stage a secondary center can result from differentiation utilizing the positional information provided by the first dominant region together with some additional asymmetry present in the differentiating system. For example, if the animal pole of the amphibian egg gives rise to the dominant or pacemaker region of the early embryo, then a secondary center arising at the dorsal lip of the blastopore could be determined by the asymmetry evident as the grey crescent in the fertilized egg.

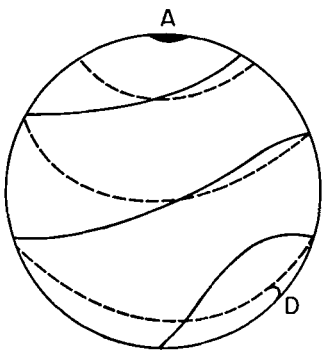

FIG. 6. Two independent sets of surfaces of constant phase difference giving two-dimensional spatial ordering. The *S*- and P_1-waves emanate from the dominant region A, and the P_2-waves emanate from the dominant region D. The solid curves represent surfaces of constant P_1S phase difference and the dashed curves those of P_2S phase difference.

We may postulate that the state of cells in this region is such that the event P_2 occurs in response to *S*, so that P_2 is non-autonomous and dependent on *S*. We suppose that only cells in the dorsal lip region can respond in this manner to *S*, but once P_2 is initiated it will propagate from cell to cell in the same way as does P_1. This property of the dorsal lip cells could be associated with some factor such as the relatively high concentration of sulfhydryl groups in this region, as shown by Rapkine (1938) and Brachet (1940). These cells then become the origin of surfaces of constant phase difference between *S* and P_2. Since these events propagate from different centers, the surfaces ϕ_{P_2S} = constant are skewed relative to one another as

shown in Fig. 6. The exact form of these surfaces will be determined by the position of the dorsal lip and by the relative rates of propagation of the S- and the P_2-events in different regions of the embryo, but they will be roughly as shown in the figure. The two sets of surfaces provide a co-ordinate grid sufficient to locate any small cluster of cells in a two-dimensional tissue. Size independence results if a critical value of ϕ_{P_1S} leads to the production of R_1 and if a critical value of ϕ_{P_2S} leads to R_2, each of which resets the appropriate phase shift, $\Delta\phi_{P_1S}$ or $\Delta\phi_{P_2S}$ in the manner described in section 4(D). For the case of the amphibian gastrula, we must assume that such regulation does occur. This will be discussed in more detail in section 7.

Three-dimensional ordering requires S, P_1R_1, P_2R_2 and P_3R_3 with three corresponding centers. A mechanism requiring wave propagation of seven events does not seem overly complex for establishing a size-independent three-dimensional co-ordinate grid in a time-ordered tissue.

The differentiation of the third center could utilize the positional information already established by the first two centers together with any further asymmetry already present in the embryo, e.g. a concentration gradient related to inside–outside asymmetry. Such asymmetry, as in the case of the use of the grey crescent asymmetry to define a second center, can be sufficient for the differentiation of a third center but may not be in itself sufficient for the establishment of fine-grained positional information.

We now have imagined a set of three independent, non-interfering centers as sufficient to establish the positional information necessary for the development of three-dimensionally asymmetric organisms. For finer detail frequency steps can be used, or more than one type of P-wave can be generated from each center. This more or less completes the general formulation of our model at the third level of abstraction listed in section 2. The model is, we hope, widely utilizable in its present form for the interpretation and prediction of results in experimental embryology at that level of abstraction. We shall make a number of interpretations, predictions, and observations relating to experimental embryology in section 7.

6. Detailed Realization of the Model

As stated in section 2, any attempt to specify in detail the biochemical mechanism and molecular species involved in the S-, P- and R-waves cannot be regarded as other than illustrative of the type of molecular process which we envisage to be involved in generating organizing waves in embryos. The concrete realization to be presented does, however, serve to bring the argument into sharper focus in relation to experimental interpretation and prediction, which will be considered in the next section.

(A) THE *S*- AND *P*-EVENTS

The S*-event*

The *S*-event has three properties which define its essential characteristics. It is (1) autonomously periodic, (2) refractory to activating signals during a substantial fraction of its period, and (3) altered in character when driven at a frequency greater than its natural frequency. The first two properties are characteristic of many non-linear self-sustaining (limit cycle) oscillations. The refractory period arises from the fact that there is a definite frequency range over which such an oscillator can be driven or entrained by a forcing oscillation of fixed and finite amplitude (see e.g. Minorsky, 1962, Chap. 18). Outside of this range the oscillator is insensitive to activating signals, until these reach sufficiently high frequencies that a new class of phenomenon, asynchronous quenching or excitation, occurs (Minorsky, 1962, Chap. 24). The frequency range we are considering for the *S*-event does not extend into this region. Thus there is nothing unusual about the refractory period, which refers to a natural property of non-linear oscillations.

The third property of the *S*-event was introduced in order to achieve the phase shifting of the *P*-event relative to the *S*-event, which necessitated an alteration in the character of the latter when it was driven at greater than its natural frequency. This alteration resulted in an uncoupling of the *S*- and the *P*-events, in a cell. There are several ways of realizing such an alteration. Probably the simplest is to suppose that the amplitude of the *S*-event is frequency-dependent: the greater the difference between the driven or entrained frequency and the autonomous frequency, the smaller is the amplitude of the *S*-oscillation. This is a natural property of limit cycle oscillations when they are operating in their continuous mode [not in the relaxation oscillation mode, wherein the amplitude is constant and independent of frequency (see e.g. Wever, 1965)]. Thus we may postulate that the *S*-event has the characteristics of a limit cycle with the above properties.

A natural biochemical candidate for the *S*-oscillation in embryonic cells is a molecular control circuit involving positive and negative feedback to give the limit cycle characteristics described above. The nature of the circuit will depend upon the period of the oscillation. If this is relatively short, up to about 15 minutes, then it is likely to be a control circuit which does not involve macromolecular synthesis, such as the glycolytic oscillation studied by Betz & Chance (1965) and analyzed mathematically by Higgins (1967). For longer periods of oscillation, up to a few hours, a control circuit involving protein and possibly mRNA synthesis is likely to be involved. Both cases are included in the control circuit diagram shown in Fig. 7. This highly schematic circuit shows a genetic locus, *G*, producing mRNA *X*, which directs the synthesis of enzyme *Y*. The enzyme transforms substrate *L* into

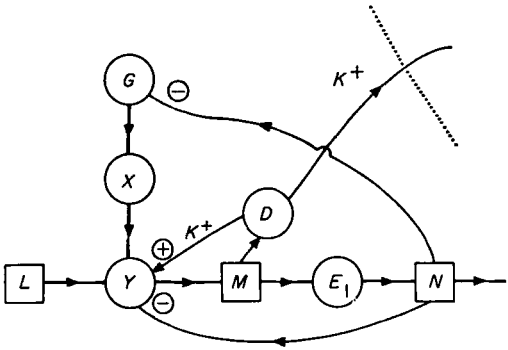

FIG. 7. Possible control circuit for the periodic *S*-event. The operation of this control circuit is described in the text in section 6(A) where the definitions of symbols in the figure can be found.

product *M*. This product has the effect of increasing the intracellular concentration of free monovalent ion, let us say K^+, which is an activator of the enzyme *Y*, thus producing positive feedback. The free K^+ could arise from release of the ion from a bound form on a macromolecule or a membrane surface, *D*. We conduct the following discussion in terms of K^+ as the ion involved in the *S*-event, but it should be emphasized that Na^+ or some entirely different molecular species could equally well be the activator and transmitter.

The metabolite *M* is converted to *N* by the action of another enzyme, E_1, and *N* is assumed to act as an inhibitor of the enzyme, *Y*. It also acts as a co-repressor if gene control is involved in the circuit, and/or as an inhibitor of protein synthesis if this is part of the control process. The potassium ion is assumed to act as the signalling substance to trigger the *S*-event in an adjacent cell, the transmission occurring via a tight junction, shown as a dotted line in the figure. Such a circuit can behave as an oscillator if the time-constants for the various steps are properly chosen. The refractory period will depend largely upon the rate of disappearance of the inhibitor substance, *N*, and the strength of its inhibitory and repressive action on *Y* and *G*, respectively.

The P-*event*

The second process involved in the phase-shift model is the *P*-event, which is normally initiated by the *S*-event. We may suppose that a simple enzyme activation is involved, although once again this could be a more complex process involving mRNA and protein synthesis and their control. The activator of the *P*-event produced by the *S*-process may be taken to be the substance *M*, product of enzyme *Y*. Let the enzyme which is activated

by M be designated Z, its substrate U, and its product the metabolite V, which is converted by another enzyme E_2 to a metabolite W. We suppose that V is a second activator of Z, while W is an inhibitor. The substance V can diffuse from cell to cell via tight junctions. The representation of this circuit is shown in Fig. 8.

It is assumed that there is a fairly sharp threshold for the activation of Z by M, which can be interpreted to mean that the stoichiometry of the activation is large (Monod, Wyman & Changeux, 1964). Then if the amplitude of the M-oscillation is below the threshold for Z-activation, the P-event will not be caused by the S-event. In this case, Z-activation will occur when the metabolite V enters the cell from an adjacent cell which has generated

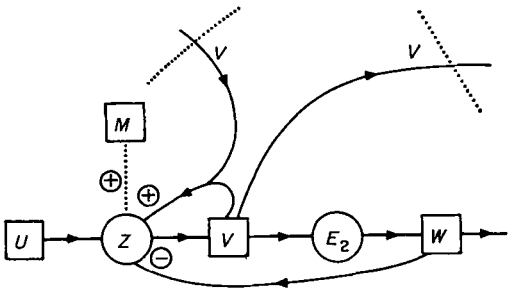

FIG. 8. Possible control circuit for the periodic P-event. The operation of this control circuit is described in the text in section 6(A), where the definitions of the symbols used in the figure can be found.

a P-event. We assume that the dynamics of the P-process are like those of a relaxation oscillator outside of its range of autonomous oscillation, so that any successful activation produces a response of fixed amplitude. In this way the reliable transmission of the V-signal from cell to cell is assured.

Given the existence of an initial frequency gradient for the autonomous S-oscillation as described in section 4(A), and the frequency dependence of the amplitude of this oscillation, it follows that there must be a progressive decrease in the amplitude of the driven or entrained S-oscillation as one moves away from the pacemaker region of the tissue. At some point this amplitude falls below threshold for the activation of the Z-enzyme, and from this point the phase-shifting of the P-event relative to the S-event will commence. Thus the extent of the organizing region, within which the phase difference is ϕ_0, will be determined by the slope of the initial frequency gradient and by the degree to which the S-oscillation amplitude is dependent upon frequency. The release of potassium by M is assumed to be another relaxation-type process, the amplitude of the K^+-signal being independent

of the amplitude of the M-oscillation over a considerable range of variation in the latter. Then reliable transmission of the S-event, and thus the establishment of a time-base for development, will occur.

The V-metabolite may be any one of a wide choice of biochemical species, the only constraint being that it should be diffusible through tight junctions. It could, for example, be a small polypeptide. Since different P-events are assumed to occur, giving different co-ordinate axes of positional information in 2, 3 and higher dimensional ordering as discussed in section 5, and must belong to some set of substances. This set may be very small, however, with possibly no more than three or four members. Although it is clear that many different informational fields must be established during the course of development in a complex embryo, the same few substances could be involved in the phase-shift mechanism. Since these substances would be produced periodically in cells which are in different initial physiological states according to the field being considered, say the limb field or the eye field, their effects on the biosynthetic potential of the cells could vary considerably with the state of the cell. If this were the case, then the mechanism for the establishment of positional information in tissues would be of a universal kind, possibly varying little even between species of organism.

On the other hand, each specific developmental field might involve a specific V-substance or set of V-substances, depending on its dimensionality, in which case there would have to be a relatively large number of different molecular species, and a correspondingly large number of enzymes (or enzyme sequences) Z. In either case, small polypeptides would have the right kind of size and specificity for the properties required of V. Since this type of molecule would diffuse more slowly than would a monovalent ion such as K^+, there would be a phase shift between the S- and P-events in cells as one moves away from the pacemaker or organizer region. This shift might be of the order of one second per cell. Longer delays could occur if the enzyme sequence, X, is long compared with that involving enzyme Y, leading to M-production and the monovalent ion pulse.

The same considerations of specificity mentioned above in relation to the P-events involved in different developmental fields apply to the S-events. Either there is one universal S-process, in which case the particular Y which is activated depends upon the state of the cell in which the S-event occurs; or else there are many different possible S-events. In the latter case, the particular S-event occurring must depend upon the state of the cell. The initial conditions of the system must enter into the determination of the developmental process in both cases, either by the specification of the S-event or the response to S via Y.

(B) PHASE-SHIFT AND MOLECULAR DIFFERENTIATION

It was stated in section 4(B) that phase differences between *S*- and *P*-events lead to differences of cell state, so that positional information leads to ordered, spatial heterogeneity of cell states. In terms of the realization of those events described above, a concrete interpretation of this process can now be given.

Monovalent ions such as K^+ affect the activities of many enzymes, among them those involved in glycolysis and oxidative phosphorylation. A potassium pulse could then result in a transiently increased synthesis and availability of high-energy compounds such as adenosine triphosphate (ATP), carbamyl phosphate (CAP), acetyl-CoA, etc. The metabolite *V* could affect the activities of biosynthetic enzymes which require the high-energy compounds for their action (as aspartate trans-carbamylase requires CAP, and choline acetylase requires acetyl-CoA). Thus the time interval between the K^+-pulse and the *V*-pulse could affect the biosynthetic activities of a cell, insofar as the sizes of the pools of high energy compounds will be periodic functions of time. Secondary consequences of such state differences can then follow.

Another interesting mechanism of action of K^+ is via cyclic AMP, now widely implicated in cellular regulatory processes (Robison, Butcher & Sutherland, 1968). Potassium chloride causes a marked increase in adenyl cyclase activity (Sattin & Rall, 1967), so that the potassium ion could have its affect via cyclic AMP, causing a wide spectrum of changes in enzyme and protein synthetic activities. The metabolite *V*, acting as a hormone-like substance, could also affect enzyme activities in a particular manner, so that the time interval between the potassium pulse and *V*-production would be very important in determining the state of a cell and its biosynthetic potential.

Both K^+ and *V* could have more dramatic affects on cell states if they can influence gene activities directly. In a dividing cell, the time of occurrence of the *S*- and the *P*-events relative to the G_1–*S*–G_2–*M* cycle of the cell could have an influence on the types and the rates of mRNA which are transcribed. An *S*- or a *P*-event occurring during the phase of DNA replication could alter gene activities in a quite different manner from the same event occurring during G_1; and the actual time in the DNA-replication phase at which an event occurs would be expected to be very important in relation to the specific gene affected. Methylation of gene bases and phosphorylation of histones or chromatin acidic proteins are processes which the *S*- and the *P*-events could influence, for example. Since the establishment of positional information frequently occurs in embryonic tissues in which there is active cell division, this direct influence of the *S*- and the *P*-events on gene

activities is perhaps the most likely manner in which phase differences can direct the course of cell differentiation.

The exact effects of these periodic events on the state of a cell will depend upon the biochemical state of the cell itself, and also upon the relationship between the frequency of the organizing waves and that of cell division. Only when these frequencies are the same or multiples of one another will the *S*- or the *P*-event occur repeatedly at exactly the same stage of the division cycle. As the cell cycle time varies, so will the effect of the organizing waves and the phase differences, so that very complicated effects in space and in time occur. It is just such ordered complexity which appears to underly the process of embryonic development.

(c) THE *R*-EVENT

It is not necessary to give a detailed description of the *R*-event, since it is a process which is essentially the same as the *P*-event It is not an autonomously-oscillatory process, like the *S*-event, but rather undergoes activation and propagation from cell to cell by means of some substance like V which activates the *P*-process. It was proposed that the *R*-event is initiated by some special phase relation of the *S*- and the *P*-events such as coincidence. We can postulate that it has a threshold of activation for both K^+ and V simultaneously. If either is below threshold, no *R*-event occurs. It is of course not necessary to require the coincidence of S and P in a cell for activation of R; this is only one possibility. Any phase angle between S and P could be postulated to activate R, the dependence of *R*-activation on K^+ and V being then different from that proposed above. V might be an inhibitor and K^+ an activator, so that the *R*-event occurs only when S and P differ in phase by some value ϕ_M.

The substance produced intracellularly by the *R*-event, which we have called Q, must have the property of reducing the phase-shift of the *S*- and *P*-events per cell, $\Delta\phi_{PS}$. We can assume that it either increases the rate of production of substance V, or that it increases the diffusion rate of V from cell to cell. In the first case the substance could be an allosteric modifier of enzyme Z, giving an increased rate of V-production. Alternatively, instead of shifting the *P*-event closer to the *S*-event, Q could cause a decrease in the rate of diffusion of K^+ from cell to cell, thus slowing down the *S*-propagation. Q could then be something as simple as free Ca^{2+}, interfering with K^+ transmission through the tight junctions. The Q species is assumed to disappear slowly from a cell, either by decay, loss across the membrane, or transformation from free to bound state. Thus a regulating system, according to this theory, involves intermittant activation of the *R*-process, to maintain some mean level of Q in the cells.

In the case of regulation of a periodic pattern, it was assumed in section 4(D) that the phase shift per cell, $\Delta\phi_{PS}$, was reduced only when m successive R events occur within a single period, T, of the developmental clock where m is the number of periods in the pattern. In order to avoid interference between R-events propagating from cells in which the phase angle between P and S has the particular value ϕ_M which activates the R-process, it is simplest to suppose that the rate of propagation of this event is greater than that of the S-event. Then the R-wave originating at one cell will have passed a cell in which the critical phase angle, ϕ_M, occurs, before the next S-event in that cell. Hence two R-waves travelling in opposite directions will never meet in a single cell. If such a coincident arrival of two R-waves at one cell did occur, the wave would cease to propagate, since cells on either side of the one receiving the double signal would be in a refractory state. The refractory period need be very short for the R-event; the time for the wave to propagate a distance of three or four cells would be sufficient, for example. A short refractory period and rapid R-wave propagation give the necessary properties for regulation of periodic patterns. Any cell will then receive m R-signals within a single period of the clock, T, m successive activations being required to bring the Q-substance to a sufficiently high level in each cell to affect the decrease in phase shift, $\Delta\phi_{PS}$, in the manner discussed above. Once again, threshold can be assumed to arise from a large stoichiometry in the reaction between Q and the event affected by it, whether S or P, resulting in a decreased $\Delta\phi_{PS}$.

In concluding this section, let us emphasize again that the specific molecular mechanisms considered to constitute the S-, P- and R-events are regarded as purely illustrative of the type of process which might operate in the generation of positional information by the phase-shift model. It would be very surprising if much of this conjecture survived experimental investigation. The argument serves primarily to show that the various events treated formally in the establishment of the properties of the system can be realized in terms of elementary and familiar biochemical processes.

7. Analysis of Selected Observations: Proposed Experiments

In this section we will show how particular embryological processes can be interpreted and analyzed in terms of the concepts which have been developed. The examples selected for analysis are typical of pattern-forming processes, and they will illustrate both the potential and the limitations of the phase-shift theory. It is evident that in most instances of pattern formation in embryos there are two distinguishable but interrelated levels of organization: a global or "field"-type process which gives to the tissue some

well-defined macroscopic order; and local interactions which determine the fine structure of the pattern. It is the former phenomenon in which we are primarily interested, since we are concerned with the ordered spatial distribution of phase information over large numbers of cells in developing systems.

(A) DEVELOPMENT AND REGENERATION IN *HYDRA*

The Hydrozoa provide in many respects model systems for the analysis of developmental mechanisms. Studies with these organisms have led to some of the most lasting and illuminating theories of embryogenesis. The whole theory of metabolic gradients, for example, originated with Child's experiments on the resistance of *Hydra* to various toxic substances, carried out in the early part of this century (Child, 1919, 1941). Since that time experimental studies with *Hydra* have given rise to various elaborations of Child's theories and the development of new ones. We will draw upon the same body of experimental data to illustrate how the phenomena can be interpreted according to the phase-shift theory, and what type of experiment can be done to test this interpretation.

It was stated in section 4(A) that the pacemaker or organizing region of an embryonic tissue is determined by the high point in a frequency gradient which is assumed to be present in the developing system. It is natural to identify this pacemaker region with the region of highest organizing potential in *Hydra*, which is the hypostomal region. We propose, then, that this region has the highest natural frequency for the *S*-event, and that the rest of the organism is drawn into synchrony with the developmental clock in this distal zone. Surfaces of constant phase then originate from the hypostome and extend to the basal disc. Since there are no periodicities along the vertical axis of *Hydra*, the phase shift from hypostome to basal disc is less than 2π (the periodicities involved in tentacle formation must be analyzed separately, a different axis being involved). Since *Hydra* consists of two cell layers, the surfaces of constant phase are annuli, two cell diameters in width (Fig. 9). The phase angle between the *S*- and the *P*-events determines which cells will constitute hypostome, which gastric region, etc. There are only five different regions of positional information along the major axis and thus only five distinguishable ranges of phase difference, according to the coarse division of *Hydra* depicted in Fig. 9.

When the hypostome and tentacles are removed, the distal end, having the highest natural frequency of the *S*-event, will again become the organizer region, imposing its frequency on all other cells and establishing the origin of the *S–P* phase shift. The phase gradient will be re-established and the organism will regenerate normally.

If a second head is grafted into some region of an entire host, then two organizing regions with high natural frequencies will be present in one organism. There is no reason for one to dominate over the other, so neither should be assimilated. There will be a tendency for both heads to become synchronized, since an *S*-event starting in one head will be transmitted to the other once the graft has taken and electrical or other communication established with the host cells. Thus any initial phase difference between the *S*-event in the two hypostomes will tend to be reduced to an interval

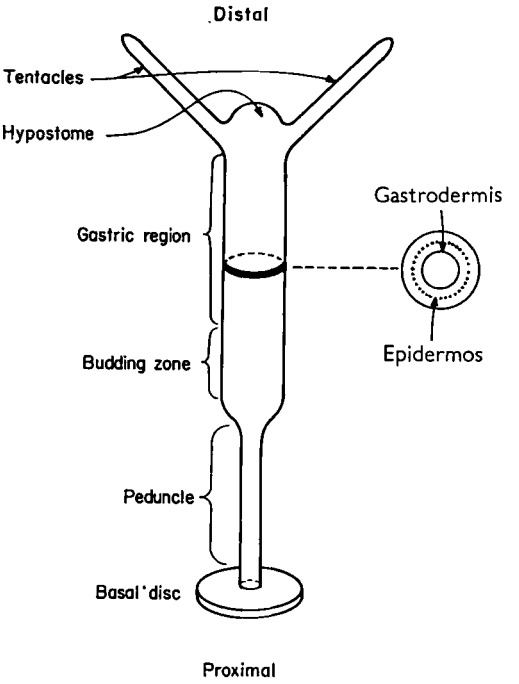

FIG. 9. Schematic diagram of *Hydra littoralis*.

determined by the time required for the *S*-event to propagate from one hypostome to the other, which will be a matter of seconds. Given that the period of the *S*-event in *Hydra* is of the order of a few minutes, which is a reasonable estimate in view of the developmental periods of four to eight hours in this organism, the result will be virtually synchronous pacemakers in the two heads. The degree of synchrony and the extent to which the two heads compete for the organization of the whole organism will depend upon the position of the grafted head. If it is grafted in the middle of the gastric region, then it will tend to interfere with the dominance of the original head

by initiating a phase-shift process at the 'wrong' point in the original organism. This could lead to some disorganization of the animal, possibly with disturbance of the normal budding process. On the other hand, if the graft is placed near the original hypostome and the resultant two-headed *Hydra* is more or less symmetrical, the two heads should co-operate in organizing the whole organism, causing little interference.

If instead of heads, sub-hypostomal regions of *Hydra* are grafted to a host organism, the graft may or may not result in the formation of a secondary head. The result depends upon the region from which the graft is taken, and the position in the host to which it is grafted. In general, tissue taken from distal regions has a greater capacity to initiate a secondary head than tissue taken from proximal regions; while distal regions of the host exert more inhibitory influence on secondary head formation than do proximal regions. According to the phase-shift theory, the capacity of a tissue to form a secondary head is determined by the natural frequency of the S-event in the cells of the tissue. This will be higher the more distal the region, hence the observed correlation with the greater developmental potential of the more distal regions. The inhibitory influence of a head on grafted tissue arises from the phase-shift induced in the graft by the pacemaker region.

Webster (1966) observed that the capacity of a region to form a secondary head in a host varies with the time that the region has undergone regeneration after removal of the original head. If only the hypostome and tentacles are removed, for example, then the sub-hypostomal tissue in the regenerating *Hydra* has within a few hours the capacity to form a secondary head when grafted to a defined region of an entire host (the peduncle was used). If more of the sub-hypostomal tissue is removed with the hypostome, the remaining region takes longer to develop the same potential for secondary hypostome formation when grafted to the peduncle. His interpretation of this result was that there are two gradients involved: a gradient of inhibition produced by the hypostome and hence maximal at the distal end of the organism and minimal at the basal disc; and a gradient of threshold for response to inhibition, again with maximum at the hypostome and minimal at the basal disc. When the hypostome is removed, the concentration of the inhibitory substance falls until it crosses the threshold level of inhibition for the remaining, distal portion of the remaining organism, when that portion will begin to regenerate. As regeneration occurs, cells differentiate into the missing parts, restoring the original gradients of threshold and inhibitors. It is presumably the restoration of the original threshold gradient during regeneration which increases the capacity of a distal region for secondary hypostome formation upon transplantation to the peduncle of another organism.

In terms of the present theory, the analogue of the threshold for inhibitors is the gradient of natural or autonomous frequency in *Hydra*, the maximum being always at the distal end of the organism. The analog of the gradient of inhibition is the phase gradient. Upon removal of the head, the original *S*-frequency is lost and a new, lower frequency becomes the dominant one. The phase gradient is re-established from this distal region and regeneration begins. In the course of regeneration, the original gradient of natural frequency gets re-established, because the distal cells differentiate into hypostomal cells, one of whose properties is a high natural frequency. Thus there is an increase in the capacity of these regenerating cells to form a secondary hypostome when grafted to a host, in view of their increased natural frequency.

The fact that the pacemaker or organizing region is determined by relative, not absolute, frequency, has a number of experimental consequences. It should be possible to reduce the frequencies throughout a whole organism by exposure to low temperature, for example, or by treatment with a metabolic inhibitor. Then a piece of sub-hypostomal tissue taken from an untreated animal ought to have a greater capacity to produce a secondary hypostome in the metabolically inhibited organism. Another expectation is that a section of the mid-gastric region of a *Hydra*, grafted with normal orientation to the distal end of an organism from which the head has been removed, should have its gradient of phase reversed. The region of highest frequency in such an organism will be the sub-hypostomal region of the host, which should dominate the graft and result in the formation of a peduncle at the most distal end of the graft. These results would be expected on the basis of any theory in which organizing potential is a relative property of the cells, not an absolute one. They are not consistent with any theory which postulates a one-way information flow in cells.

A more direct test of the theory is as follows. If one could introduce a signal into the gastric region of *Hydra* between the hypostome and the budding region which initiated the *S*-process and had the same frequency as that of the pacemaker, it would act as an organizing center and induce the formation of a new head. One way of introducing such a signal would be to implant a micro-electrode in this region and introduce a short pulse of potassium once every few minutes. Since the *S*-event frequency is not known (still less, the existence of such an event), it would be necessary to carry out a frequency search to see if there is some range which produces the desired response: initiation of a secondary head. The periodic pulsing would have to continue for several hours in order to establish the new pacemaker, 12 to 24 hours being a reasonable estimate in view of the developmental time-scale of *Hydra*.

Instead of using a microelectrode to introduce an electrochemical signal into a cell, it could be used to pick up a signal propagating through the cell. In order to detect such a signal, it would be necessary to have some estimate of the expected magnitude of the ionic flux, in order to set up the conditions for maximal signal-noise ratios. A direct observation of a periodic signal in a cell would of course be very convincing evidence for a theory of the type being proposed.

Maintaining a microelectrode in position for this period of time is a major difficulty in an organism like *Hydra*. An alternative procedure is to run a fine wire through the organism in the region between the hypostome and the budding zone at right angles to the vertical axis of the organism. A second wire is then placed in the *Hydra* medium outside the organism to complete the circuit. If the wire passing through the animal is insulated except where it passes through the organism, a pulse of current through the circuit will be carried electrolytically through the cells and could activate the *S*-process. If so, then a particular frequency and voltage may be found which induces the formation of a new head in the region which would normally be inhibited by the original hypostome. Such experiments are in progress.

The occurrence of a budding zone in the lower gastric region of *Hydra* is a phenomenon which does not have an obvious interpretation in terms of gradient theories. The influence of the hypostome in preventing secondary head formation in an animal extends to the peduncle, since an isolated peduncle is capable of regenerating a complete *Hydra*. This influence has generally been interpreted in terms of the production by the hypostome of an inhibitor of secondary hypostome formation. However, within this domain of inhibition, budding occurs. A bud is an apparently autonomous aggregate of cells which organize themselves into a new *Hydra*. Evidently they have escaped from the influence of the parent hypostome. In terms of gradient theories, it is necessary to postulate the occurrence of a secondary gradient arising from the peduncle and somehow neutralizing the hypostomal inhibitory influence in the budding zone, but only in this zone. Thus it is the ratio of concentrations of the two substances which determines where budding will occur.

In terms of the phase-shift theory, the simplest and most natural explanation of the budding process is that the budding zone cells are induced to have a high frequency of the *S*-event, too high either to be entrained by or to entrain the rest of the parent organism. This high frequency could be caused by a particular phase angle between *S*- and *P*-events in cells of this region, in the same way that other inductive processes are assumed to depend upon ϕ_{PS}. Alternatively, there could be a second *P*-event which originates in the peduncle and propagates toward the hypostome, creating a second

gradient of phase difference. This second gradient could then give rise to the particular phase conditions which create a region of high *S*-frequency in the budding zone. Cells with the high frequency will entrain one another, and the cells with highest local frequency will constitute the pacemaker or organizer of the bud. Since the high frequency region (let us suppose the frequency to be about twice that of the parent hypostome) has limited spatial distribution, only a limited number of cells will be entrained. Once they have become organized into a unit, they will remain autonomous and develop independently so long as their frequency remains outside the range of entrainment of the parent pacemaker; and this autonomy will be ensured when electrical communication with the parent is lost at some stage in bud formation.

If this hypothesis is valid, then it should be possible to induce the formation of a bud by introducing into any region of *Hydra* the correct high frequency of ionic or electrical pulsing. This might be expected to be about twice that of the primary head pacemaker, if such exists. However, it is probable that high frequency alone is not sufficient to initiate bud formation, this process being limited to regions of the organism which are competent for reasons additional to their potential for high frequency of *S*.

(B) POSITIONAL INFORMATION IN THE AMPHIBIAN EMBRYO

In section 4 we considered the manner in which two axes, defined in relation to surfaces of constant phase, could be set up in terms of events propagating from the two centers which have always been recognized to be of primary importance in the spatial organization of the amphibian embryo, the animal pole and the dorsal lip of the blastopore (Fig. 6). We want now to attempt an interpretation of some of the experimental observations on early amphibian development in terms of the phase-shift theory, and to suggest some experiments for testing its validity.

The first clear evidence of differential developmental potencies in different regions of the amphibian embryo emerges in the early gastrula. Holtfreter (1938) showed, by isolating fragments of tissue from early urodele gastrulae and culturing them in balanced salt solution, that the type of tissue into which such fragments could differentiate was related in a well-defined manner to their position in the gastrula from which they were taken, even though there was no cytological evidence of differentiation at the time they were isolated. According to the phase-shift theory, such spatially distributed cell states must arise in relation to surfaces of constant phase, and so it is interesting to see if there is a correspondence between the surfaces shown in Fig. 6 and the regions described by Holtfreter as possessing different developmental potentials. Figure 10(a) is the same as Fig. 6, with the surfaces

numbered for reference. Figure 10(b) is a schematic rendering of Holtfreter's results, showing the distribution of the three distinguishable regions, generalized epidermis (neural plate and epidermis are not distinguishable at this stage), chordamesoderm, and endoderm. From a comparison of these figures, it is evident that the boundary separating chordamesoderm from epidermis corresponds roughly to phase surface ϕ_{P_2S} number 2. This suggests that positional information provided by the ϕ_{P_1S} surfaces, originating from the animal pole, is not relevant for the specification of the boundary between ectoderm and mesoderm, whereas that arising from the dorsal lip in the form of the ϕ_{P_2S} surface is. On the other hand, the most ventral region

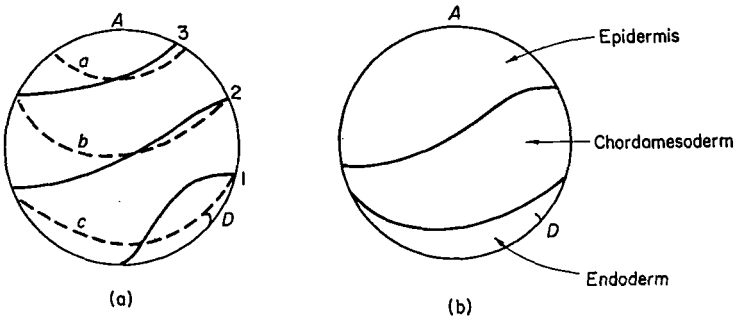

FIG. 10. Comparison between surfaces of constant phase difference expected in the early amphibian gastrula on the basis of the present model, (a) and (b) the regions described by Holtfreter as possessing different developmental potentials on the basis of experiments with early urodele gastrula (1938). Here A indicates the animal pole and D the dorsal lip. In (a) it is assumed that S and P_1 propagate outward from A giving rise to the dotted surfaces of constant phase difference ϕ_{P_1S} labeled a, b and c, and that P_2 propagates outward from D giving rise to the solid surfaces ϕ_{P_2S} labeled 1, 2 and 3.

of ϕ_{P_1S}, lettered c, corresponds to the boundary between chordamesoderm and endoderm. Thus a particular phase angle between S and P_1 could provide the positional information required for this discontinuity. If this interpretation of the manner in which the earliest manifestation of positional information in the amphibian embryo comes about is correct, then it is clear that at this stage of development the informational instructions are particularly simple.

As gastrulation proceeds and tissue invaginates, the various regions of the gastrula become more fully determined with respect to their developmental fate, their relative positions change, and the regions shown in Fig. 10(b) become subdivided. There is clearly a rapid and complex development of secondary centers or pacemaker regions and fields in the subsequent

development of the amphibian. This requires detailed study and interpretation in terms of the phase-shift theory. At this point we will restrict our attention to the analysis of experiments illustrating the properties of the earliest embryonic field, that arising in relation to the primary organizer.

The importance of the dorsal lip of the blastopore as an organizing region in the early amphibian embryo was fully established by the classical experiments of Spemann & Mangold (1924). Transplantation of tissue from this region to another part of an early gastrula results in the induction of a second site of invagination and the appearance of a secondary embryonic axis. It is of some interest to consider how this could come about in terms of the phase-shift theory.

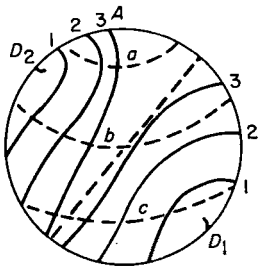

FIG. 11. Surfaces of constant phase difference to be expected when dorsal lip tissue D_2 from a second gastrula is grafted at about the time the primary dorsal lip D_1 appears. The dotted line represents the boundary between the phase patterns ϕ_{P_2S} emerging from D_1 and D_2.

Suppose dorsal lip tissue to be grafted to the region marked D_2 in Fig. 11. This will result in the initiation of a second origin of the P_2-event, which will propagate away from this region and establish surfaces of constant ϕ_{P_2S}, as does the original dorsal lip tissue. Both P_2-events will have the same frequency as S, since they are dependent upon the occurrence of the latter. There will be a region of the embryo where the two propagating P_2-waves interfere with one another and arrest further propagation. This interference is due to the existence of a refractory period in cells after the occurrence of P_2. Thus the domains of dominance of the two centers will be separated by a surface located between them. The exact position of this separating surface will depend upon a number of factors, a major one being the time at which the secondary dorsal lip is grafted. If this is done at about the same time as the primary dorsal lip begins to appear, then the domains of dominance of the two centers should be about equal. However, if the primary center is allowed to act and establish a wave-field over the whole embryo before the second center is grafted in, then the latter will have a more

restricted domain of dominance and the resultant secondary axis should be reduced in size. Independently of their relative sizes, each center should produce a complete set of phase-surfaces, ϕ_{P_2S}, assuming that there is a regulation event which sets the phase-shift per cell in the manner discussed in section 4.

Whether or not a complete and normally-proportioned embryo results from the secondary set of surfaces ϕ_{P_2S} will depend upon the position of the graft in relation to the animal pole, and hence upon the interaction of these surfaces with the surfaces ϕ_{P_1S} = constant. If the graft is placed very close to the animal pole, then there should result a relative preponderance of ectodermal and mesodermal structures over endodermal ones in the secondary embryo, since relatively little of the tissue lying below the lowest ϕ_{P_1S} surface, corresponding to presumptive endoderm, will be included in the secondary field. The closer the graft is placed to the normal meridian of the dorsal lip, the more complete should the secondary embryo be.

Figure 11 shows the surfaces of constant phase which would be expected in a gastrula which has two organizers, the original one marked D_1 and a secondary one, D_2, grafted into the host at the late blastula stage, let us say. The line of division of the domains will be sharp if the refractory period of the P_2-event is long, and blurred if it is short; i.e. the degree of interpenetration of the two fields is determined largely by the duration of the refractory period. The secondary embryo resulting from such a graft should be somewhat deficient in endodermal structures, since little of the presumptive endoderm is included in the secondary field.

Another important factor entering into the completeness of an induced secondary embryo is the position of origin of the graft. Spemann (1931, 1938) and Holtfreter (1938) showed that there is a definite distribution of inductive potencies in tissue from the urodele gastrula, and they distinguished especially "head" organizer from "tail" organizer. The former tends to induce heads, the latter tails. This property of inducing tissue is easily understood in terms of the phase-shift theory in relation to the initial phase-angle established by the P_2 pacemaker region between the S-event and the P_2-event. In tissue taken close to the dorsal lip of the blastopore, which acts as head inducer, we assume that the physiological conditions are such that there is a relatively short time interval between the occurrence of S and the resultant occurrence of P_2, making $(\phi_{P_2S})_0$ small. The further away from the dorsal lip the tissue originates, the longer is this time interval until one leaves the zone where P_2 occurs in response to S and no organizer action occurs. Clearly the tissues which arise in relation to surfaces of constant ϕ_{P_2S} (ectoderm and chordamesoderm) will vary in proportion to the initial phase angle between S and P_2 in the organizer tissue. The greater this angle, the less the presump-

tive chordamesoderm, hence the less the anterior neural structures induced by this tissue and the greater the posterior, tail structures.

There is one final important set of experimental observations on primary induction which we will interpret in terms of the phase-shift theory. This is the very extensive analysis of the organizing properties of dorsal lip and other inducing tissues or substances, which resulted in the distinction made by Waddington & Schmidt (1933) between "evocation" and "individuation". The former is defined as the induction of cytodifferentiation in embryonic cells, without any necessary spatial order between the differentiated cells; while the latter refers to the process whereby a spatially-organized tissue or organ or whole embryo comes into being as a result of an induction. According to our theory, the latter process would occur whenever the initiation of an organizing wave, such as a wave of the P_2-event, occurs in a spatially localized region, the initiation center, such as the dorsal lip or a graft. For then the waves would spread out in a spatially-ordered manner from the center, primary or secondary, an axis would be established, and organization would result. This is the observed response to any spatially-localized induction, whether by living organizer tissue, adult liver, methylene blue-impregnated ectoderm, or other inducing stimulus (Waddington, 1954). But if the stimulus is not spatially restricted, for example if gastrula ectoderm is isolated in an inducing solution, then differentiated structures arise but there is no spatial organization in the tissue: the differentiated tissues are chaotically arranged relative to one another (Holtfreter, 1951). The inducing stimulus has, we would say, initiated organizing waves at random in the gastrula ectoderm, and the result is interacting fields which organize slightly within their domains of actions but no overall coherent order. The latter behavior Waddington refers to as evocation. Our interpretation is that individuation occurs when an O-wave is initiated in a spatially-localized region of embryonic tissue, and that evocation results if the inducing stimulus is not so localized. Individuation would thus be expected to accompany any normal spatially localized embryonic induction, as it does. In particular, the reason why the primary organizer has the capacity to organize the future development of the embryo is simply that it is localized in a particular region of the gastrula, that part known as the dorsal lip.

Experimental investigation of the theory as applied to the amphibian embryo would involve procedures very similar to those involved in *Hydra*. The most direct evidence for the theory would be the observations of periodicities in late blastula and early gastrula cells, either by microelectrode, intracellular recording, or by macroelectrode, extracellular recording. More conclusive still would be an interference with the embryonic field and resultant embryo formation by the introduction of specific perturbing

frequencies, electrical or ionic, at particular positions in the embryo. If such periodic signals initiated the S-wave, then the ϕ_{P_2S} contours could be altered in a predictable manner according to the position of application of the periodic stimulus. If the P_2-event could be initiated, then secondary axes could be produced in specific spatial relation to the site of initiation. The significant, novel feature of such experiments is the prediction that the responses observed, if any, should be frequency-dependent, and that specific alterations in the embryonic field should result from the application of the periodic stimulus at specific sites in the embryo.

(c) THE RETINAL-TECTAL PROJECTION OF THE VERTEBRATE VISUAL SYSTEM

Having considered two relatively simple developmental systems, we will now turn to an altogether more complicated embryological process. An explanation of the process whereby retinal neurones make synaptic connections with specific regions of the optic tectum in vertebrates requires a refinement of the positional specification process to an extent which must tax the plausibility of almost any model. It is precisely such a problem, however, which provides a test for a developmental theory. In attempting to delineate lines of analysis for this problem in terms of the phase-shift theory, we are necessarily pushing this theory hard, and in so doing we will at times appear to go beyond the realms of biochemical plausibility. However, we believe that there are in fact good biochemical bases in allosteric behavior and the responses of multimeric enzymes and enzyme systems for the kinetic responses necessary to explain neuroembryological phenomena in terms of the phase-shift theory, although we will not spell them out in detail.

The core of the retinal-tectal projection problem appears to reside in the directional growth of retinal fibers in response to information received *locally* on or near the surface of the optic tectum. The experiments which establish this property of the retinal neurones most dramatically are those of Delong & Coulombre (1967), although this feature is implicit in the earlier studies of Sperry (1963), Gaze & Jacobson (1963), and Gaze, Jacobson & Szekely (1963). In the experiments of Delong & Coulombre, pieces of chick embryonic retina were grafted directly to regions of the optic tectum. After several weeks, the tissues were fixed and stained and the direction of outgrowth of retinal fibers from the graft were observed and analyzed. It was established that the retinal fiber outgrowths occurred in well-defined directions, dependent upon the origin of the piece of retinal tissue and its position in the optic tectum. The directions of outgrowth were all consistent with the normal retinal-tectal maps established previously, i.e. fibers were

growing in the "correct" directions to make synaptic connections with the "correct" tectal neurones.

It had been established in the earlier studies of Stone (1948) and Szekely (1954) that there are two axes of positional information on the two-dimensional surfaces of tectum and retina, which have been interpreted as two gradients (Gaze, Jacobson & Szekely, 1963). These axes are easily established on the phase-shift theory in the same way as the two-dimensional axes of the amphibian gastrula were postulated to arise. However, such axes do not tell us how it is that a retinal fiber can decide, on the basis of local information, the correct direction in which to grow. While formally it is possible to compute an angle from two co-ordinates, such a formal solution gives us no insight into the physiological and biochemical mechanisms which are at work in orientating the fibers. At the cellular level, the problem is one of directed growth control, followed by the establishment of synaptic connection. This appears to be the basis of all neuroembryological pattern formation; and an analogous process is probably involved in learning.

(D) POSITIONAL INFORMATION IN RETINA AND TECTUM

We assume that retina and tectum have matching dimensions of positional information, as is necessary for a mapping to be established. The degree of resolution of the mapping is not known in detail. It seems unlikely that the resolution reaches the single cell, although the phase-shift theory does provide this potential, as will be discussed later.

We assume that retina and tectum have initial frequency gradients along primary axes (nasal-temporal in the retina, caudal-rostral in the tectum), which result in the frequency-step pattern of Fig. 12. Within each region of constant frequency there is a gradient of phase between the events S and P_1. Along this axis, each cell is specified by a frequency, ν_i, and a phase angle, $\phi^j_{P_1S}$. A second axis is established by a second event, P_2, which originates in cells of the medial tectum and propagates laterally, let us suppose; while in the retina there will be a corresponding phase gradient running ventro-dorsally. A cell in either retina or tectum will then be specified by a frequency, and two phase angles. The co-ordinate system is shown for the optic tectum in Fig. 12. The frequency gradients correspond to the primary gradients in the diagrams of Gaze *et al.* (1963). It is necessary to distinguish between the co-ordinates in the tectum and those in the retina, so we use superscripts t and r, respectively. Thus a cell in the tectum is specified by a frequency, ν^t_i, and two phase angles, $\phi^{tj}_{P_1S}$ and $\phi^{tk}_{P_2S}$; while in the retina these co-ordinates are ν^r_i, $\phi^{rj}_{P_1S}$ and $\phi^{rk}_{P_2S}$. The events S, P_1 and P_2 are not necessarily the same in the two tissues; in fact, we will generally assume that they are distinct. Since each cell is specified by 3 variables, there is some redundancy

of information for a two-dimensional mapping. The way this information may result in a specific projection will be discussed shortly.

The trophic factors involved in the growth of the optic nerve to the tectum and its division into upper and lower branches will not be considered. We

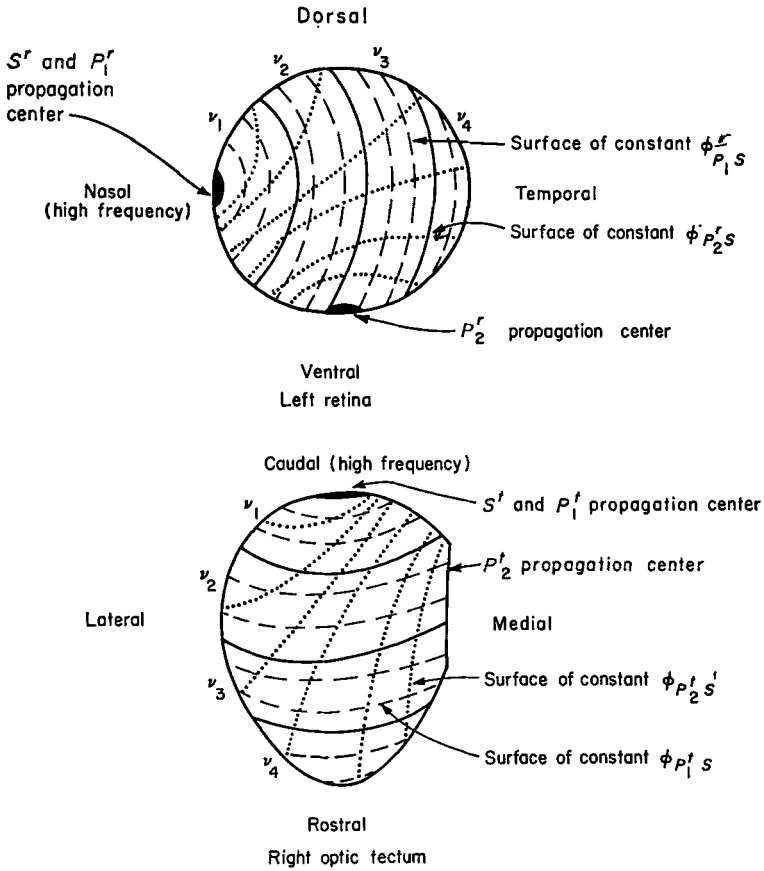

FIG. 12. Two-dimensional co-ordinate grids for left retina and right optic tectum of amphibia in 1–1 correspondence. A combined frequency step and phase gradient scheme is used for the nasal-temporal axis in the retina and for the corresponding caudal-rostral axis in the tectum; the frequencies in the various steps are indicated as $\nu_1 \ldots \nu_4$. A phase gradient scheme is used alone for the second axis.

are concerned here only with the behavior of retinal fibers when they are in the neighborhood of the tectum, say 5 to 10 μ from its surface. These fibers are then able to respond to signals coming from tectal cells. Since the problem is that of directed growth of retinal fibers, we assume that the signals coming

from the tectal cells exert their influence on retinal fiber membranes, either sensitizing them to produce pseudopodal protrusions, or inhibiting this process. We assume that if a particular signal has the effect of stimulating the formation of a membrane process at one part of a retinal fiber, then other parts of the fiber are inhibited from producing such a process, as is common in pseudopod formation. Conversely, a signal which inhibits membrane process formation in a local region of a retinal fiber will increase its probability of formation elsewhere. We will conduct the discussion in terms of the diffusion of substances from tectum to retinal fibers, but this is not the only method of signal transmission which could operate between the cells.

The first problem is to use the frequency relations of retinal and tectal cells to generate directional signals. Formally, if the frequency relations between a retinal fiber and the tectal cells in its neighborhood are such that $\nu_i^r > \nu_m^t$, considering a cell in the ith frequency band of the tectum and in the mth frequency band in the retina, then it is required that such a fiber grow in the direction of higher frequencies in the tectum, on the assumption that frequency matching occurs. Since there is a whole band of tectal cells with the same frequency, and similarly with the retina, matching this bit of positional information gives only a coarse mapping. Within this coarse map is the fine phase gradient map, which will be considered later.

To achieve frequency matching, we propose the following relations. Suppose that the S^t-event involves the production of some substance σ_0 which has the following properties: (1) it initiates the S^r-event in a retinal neurone if the neurone is in a receptive (non-refractory) state; (2) if present in high concentration relative to the concentration of a substance ρ_0 produced in association with the S^r-event in the retinal neurone, it inhibits membrane process formation in the retinal fiber; (3) if present in low concentration relative to ρ_0, it sensitizes the membrane to pseudopod formation; (4) if present at an intermediate concentration (ratio $\sigma_0/\rho_0 \sim 1$) there is no effect on the membrane; (5) the substances σ_0 and ρ_0 have half-lives about equal to the shortest period of S^t (and hence also of S^r).

The result of these properties is for the retinal neurone to grow towards the frequency band of the tectum which matches its own frequency, and there for its S^r-event to become synchronized with the S^t-event. For example, a low-frequency retinal fiber which finds itself in a high-frequency tectal region will experience a high concentration of σ_0 relative to ρ_0, since σ_0 is produced with every S^t-event. Furthermore, it is that part of the retinal neurone closest to the S^t-propagation center which experiences this inhibition first with every S^t-event, since a diffusion wave of σ_0 will travel along behind the S^t-wave front and will reach this side of the retinal fiber before it reaches the other. There will then be a tendency for pseudopod formation to occur

on the opposite, uninhibited side of the fiber, and so growth will occur away from the S^t-propagation center, down the frequency staircase. Exactly the opposite situation holds for a high frequency retinal fiber in a low frequency region of the tectum, in view of property 3. Property 5 is required so that the substance σ_0 will be present in higher mean concentration in a high frequency tectal region than in a low-frequency region, and similarly for ρ_0 relative to different frequency regions in the retinal fibers. There is a considerable range of half-lives which will give these results.

The vectorial element in the retinal fiber growth response is due to the directed propagation of the S^t-event, together with the membrane response characteristics, in much the same way as directed migration of myxamoebae results from periodic propagation of acrasin from the aggregation center according to Shaffer's (1962) theory of slime mould aggregation. The directed growth of retinal fibers as described above is a very similar process. The added refinement that retinal fiber growth can occur in either direction relative to the $S^t - P_1^t$ axis depends basically upon the fact that we are dealing with a two-component system with both signallers (tectal cells) and seekers (retinal fibers) possessing a frequency. The result of their interactions as described by properties 1 to 5 is for retinal fibers to grow to regions of the tectum with a frequency of O-waves which matches their own, and for the S^r- and S^t-events to become synchronized.

In order to achieve a finer mapping of retinal fibers onto tectal neurones, it is necessary to consider now how the phase gradients may be used to control the direction of growth of a fiber. We postulate that the P_2^t-event, which propagates from medial to lateral tectum, involves the production of a membrane-sensitizing substance similar to σ_0 which we call σ_2. If P_2^t is phase-advanced relative to P_2^r, assume that σ_2 acts as a trophic signal, so the retinal fibers grow towards the pacemaker region of the P_2^t-wave. If P_2^t is phase-delayed relative to P_2^r, then σ_2 has the reverse effect: it acts as an inhibitor of membrane process formation. Thus the temperal relations between biochemical events determine the nature of the response, as we have been assuming throughout this paper. We may interpret this in the above example to mean that the P_2^r-event involves the production or release of some substance, ρ_2, which when combined with σ_2 produces an inhibition of membrane outgrowth; whereas σ_2 acting in the absence of ρ_2 stimulates such outgrowth. The half-life of the substance ρ_2 would determine the duration of the inhibitory phase of the retinal fiber cycle. Once again, there will be some particular phase angle between P_2^t and P_2^r wherein the two opposing stimuli will balance one another in probability so that there is no net tendency of the fiber to move one way or the other relative to the P_2^t axis.

We now use exactly the same argument for phase-matching along the other axis, postulating the existence of another membrane-sensitizing substance σ_1, produced as a result of the P_1^t-event in the tectal cells, and a modifier of its action, ρ_1, produced as a result of the P_1^r-event. If P_1^t occurs before P_1^r, σ_1 acts as a stimulator of membrane-process formation in the retinal fiber and the fiber tends to grow towards the P_1^t-organizer region, whence the P_1^t-event propagates. But if P_1^t occurs after P_1^r, then the combined substance $\sigma_1\rho_1$ acts as an inhibitor, so that membrane processes form on the opposite side of the fiber from the P_1^t-organizer region. An equilibrium position again exists for such a system.

When there is no tendency for a retinal fiber to produce a membrane outgrowth in any preferred direction tangential to the tectal surface, we may suggest that it will tend to grow down into the tectum. Upon making contact with a tectal neuron it may be postulated that the retinal fiber will form a synaptic connection. Thus a retinal-tectal map may be realized. The degree of resolution of this map depends upon the sensitivity of the postulated signalling system to phase and frequency differences between the cells.

It should be observed that the direction of growth taken by a retinal fiber on reaching the tectum will be the net resultant of three independent signals, all affecting membrane process formation. Before frequency-matching is achieved as a result of directional growth along the frequency gradient, the phase-signalling system will produce a variable response with respect to direction, since phase relations will vary as a result of frequency mis-match. This will produce a slightly meandering growth of the fiber relative to the medio-lateral axis of the tectum, the direction of growth along this axis getting more definite as the frequency match improves.

The postulated system of retinal-tectal interaction by means of different growth-promoting and inhibiting signals implies a fairly complex control system for the regulation of neurone fiber growth. Of course, if positional information in retina and tectum does not involve a frequency staircase and is generated solely by two phase gradients, ϕ_{P_1S} and ϕ_{P_2S}, then only two of the three postulated sensitization and inhibition systems need operate. There is no direct evidence for directional growth control substances of this type operating in neural tissue, although the complexity and the specificity of pattern in the central nervous system demands the existence of some such mechanism. The ones proposed are no more than an illustration of the interactions which could give to the cells the required properties of directed growth, based upon the assumption that frequency and phase-matching provide the essential mechanism of recognition between retinal fibers and tectal neurons.

This basic recognition process whereby specific connections are established

in the central nervous system can evidently be made the basis of long-term aspects of the learning process in the adult brain. The making and breaking of synaptic connections, and changes in synaptic thresholds, can be readily understood as modifications of the same fundamental mechanism of recognition and connection postulated for the embryonic development of the brain. Frequency and phase relations between interacting neurones can be used to strengthen or weaken synaptic connections by postulating that these variables affect the types of molecule made in cells and their rates of synthesis, as has been argued throughout this paper. In fact, the whole of the phase-shift theory may be regarded as a particular type of neural-network model which exploits a natural and elementary temporal to spatial mapping in non-linear oscillatory systems. The inverse of this mapping, from spatial ordering to temporal ordering, hence behavior, is equally easily realized.

We have pointed out that the potential informational-capacity of a system ordered temporally and spatially by a phase-shift process is high, being limited only by the phase resolution which can be attained by biochemical processes within cells. We have considered so far in detail only two-dimensional informational axes, but there is no theoretical limit to the number of dimensions which can be introduced. This is because, as a tissue differentiates in response to one or two axes, new P_i-events can be initiated in any region, thus starting a new degree of phase-information. The retinal-tectal mapping required only a limited co-ordinate system relating specifically to the directed growth of retinal fibers. More informational co-ordinates are undoubtedly required for the establishment of the full functional capacity of this part of the visual system.

However, other tissues appear to require, *ab initio*, more degrees of positional information. For example, if the stem-line theory of immunological development is correct, then it is necessary that the immunopoietic tissue have within it the ability to produce of the order of 10^5 very similar but distinguishable states in order that some 10^5 very similar genes be distinguished with respect to the control of their activity. This type of resolution is possible by the phase-shift mechanism, but probably only by using a relatively high dimensionality of positional information. With only two axes, 10^5 distinguishable cell states would require a grid with some 333 distinguishable phase regions along both dimensions. The phase resolution would then have to be accurate to $(\frac{3}{3}\frac{6}{3}\frac{0}{3})$ or slightly greater than 1° between S-, P_1- and P_2-events. If the period of S is 10 minutes, then the time resolution is $(\frac{6}{3}\frac{0}{3}\frac{0}{3})$ or a little less than two seconds. It seems unlikely that this order of time difference between two events in a cell could be detected as a significant difference of temporal state. A lower limit to this difference would probably be about 20 sec. To achieve the resolution required, either the period of S

would have to be of the order of 100 minutes, which seems unreasonably long since there must be many ticks of this clock within significant developmental time periods; or there must be more dimensions of positional information, hence more *P*-events. The exact number of these depends upon what different combination of P_i-sequences the cell can distinguish. The greater the number of *P*-events, the greater the number of permutations among them, this number rising factorially. So this method of establishing positional information in a tissue has a great potential resolving power, bringing complex embryological patterns well within the domain of comprehension. The potential informational capacity of temporal codes and their use in embryological processes has been discussed previously, but in much less detail (Waddington, 1965; Goodwin, 1965, 1967).

In concluding this section, we express the hope that the large amount of speculative material presented will help to clarify rather than confuse the details of the phase-shift theory, and show how it can be applied to the analysis of specific embryological problems. Many predictions can be drawn from this analysis, some of which have been presented. The major predictions are the occurrence of detectable periodicities in embryonic cells, and the interference with embryonic processes by the introduction of specific frequencies into developing tissues. This interference should not be simply destructive; it should in fact be possible to reorganize embryonic axes by the local application of periodic signals.

8. Conclusions

The barrier to progress in the study of embryonic fields and pattern formation seems, at the present time, to be more a conceptual than a technical one. The belief that an understanding of global embryological problems will come with the application of the ideas and techniques of molecular biology to embryonic systems is, in our opinion, ill-founded. These ideas and procedures will be invaluable once we know what to look for; but they cannot tell us what type of cellular phenomenon may be involved in the establishment of embryonic axes. Molecular biology and biochemistry do, in fact, tend to reinforce the deep-seated prejudice that differences of cell state are due to differences in the permanent, as distinct from the transient, biochemical composition of cells, a prejudice which may in fact be a serious block to conceptual advance in developmental biology. Our model makes clear the fact that, whereas at some stage of differentiation it is necessary for cell-specific substances to be synthesized, it is perfectly possible that the initial stage of the differentiation process involves differences of cell state which are strictly temporal. Looking for constant differences in the biochemical

composition of cells in different parts of an early embryonic field of the type we have described would be fruitless.

The model we have presented is illustrative of a class of theories in which information is carried by temporal relations between events, spatially ordered in relation to certain initial and boundary conditions in the system. As such, it belongs to the category of theories which are of the neural network type. The parallels between the properties of our model of the epigenetic process and the behavior of the central nervous system are in fact very suggestive, and encourage the point of view that neural organization may be regarded as an elaboration and refinement of a communication process which is basic to the earliest spatial and temporal organization of the embryo.

The possible value of considering this kind of model is that it suggests a rather different experimental approach to the embryo than the current emphasis on column fractionation of tissue extracts. The investigative procedures in section 7, involving periodic local stimulation of embryos by electrical and ionic pulses, or attempts to detect such signals in embryonic cells and tissues, are again reminiscent of neurophysiological techniques. Preliminary results of studies carried out on *Hydra littoralis* in which periodic electrical stimulation was delivered locally by means of a thin platinum wire passing through the tissue of a regenerating animal have shown that a well-defined alteration of the regeneration field can be obtained using a frequency of one pulse every 2·5 minutes. The stimulation results in the induction of a secondary foot towards the distal end of an isolated mid-gastric region of hydra. The response is strongly frequency-dependent, showing that a resonance phenomenon is occurring. Such a response frequency suggests that the electrical signal may be causing the activation of an enzyme control circuit of the type described in Fig. 7, where the feedback is to the enzyme, not to the gene. The glycolytic oscillator described by Betz & Chance (1965) had an *in vitro* period of about one minute and involved, according to their analysis, the periodic activation and inhibition of phosphofructokinase, which behaved as our enzyme *Z*. The stimulus used in the hydra studies was a 1·5 v d.c. pulse lasting 150 msec, and the duration of the treatment was 24 hours. The total duration of current flow was only about 1·5 minutes, so it is clear that the response could not be due to migration of cells or the orientation of dipoles in an electric field. A full description of these results is in preparation (Goodwin, to be published).

These observations encourage further investigations of embryonic fields by similar methods. While our detailed description of the molecular basis of such periodicities in embryonic systems as presented in section 6 may be entirely wrong, the formal properties of the model may still be useful for the design and interpretation of experiments which are based upon the

assumption that periodicities are involved in the formation of developmental axes and the establishment of positional information. At a more general level, we hope that the model will be received as an attempt to analyze the developmental process in terms of cellular processes which give rise to quantitative gradients of a rather different kind from those normally considered to carry information in embryos, namely gradients of substance, thus directing attention to alternative possibilities. The model may be regarded at this level as a contribution to a more conceptually-orientated discussion of epigenesis which has been stimulated by Wolpert's (1969) interesting and provocative analysis of positional information in development.

We would like to acknowledge our indebtedness to Professor C. H. Waddington for his conviction that theoretical discussions of biological problems are necessary and useful, and to his energy in organizing the symposium at which the model presented in this paper was inspired. We are grateful to Professor Lewis Wolpert and Drs Gerald Webster and Anthony Robertson for many informative and stimulating discussions.

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APPENDIX

Wave Equation and Boundary Conditions for Organizing Waves in Tissues

The organizing waves described in sections 3 and 4 are very different from the wave motion ordinarily encountered in physics. The time courses of the concentrations of molecular species during a given event, S, P or R, may be very complex and in some cases of durations comparable to the period. However, what is important for the wave propagation is the time course of the signal. In all three waves, the concentration of signaller, Σ, may be supposed to have the time course shown in Fig. 13(a). We may use instead the idealized time course of Fig. 13(b) without loss of generality. Thus, with regard to signalling, which we may take as the essential aspect of the

event in relation to its propagation, a cell is either off, or it is on during a time τ. In contrast to ordinary classical wave motion, one is dealing with a discretely valued and not a continuously valued wave amplitude.

Because the signal from a single neighbor is sufficient to initiate an event in a cell, the cell response is the same regardless of the number of neighbors signalling at a given time. Again in contrast to classical wave propagation, there is no superposition principle for the wave amplitude.

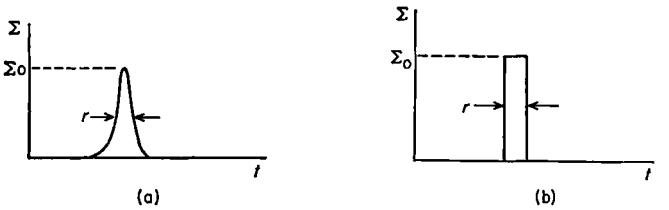

FIG. 13. Proposed time courses of signaller concentration Σ during an *S*-, *P*- or *R*-event: (a) actual and (b) idealized. The duration τ in (b) may be chosen equal to the width at half maximum in (b) or may relate to a threshold value of Σ in (a). It should be short compared to the period *T*.

The boundary conditions ordinarily occurring in wave propagation problems are homogeneous, involving linear combinations of the wave amplitude and its normal derivative according to the extent of reflection and transmission at the boundary. Here the refractory period completely eliminates reflection, and transmission cannot occur because the boundary coincides with the boundary of the embryo or with a boundary of a tissue across which there is no communication.

It should be clear from the above three paragraphs that while classical wave theory may be a fruitful source of analogies, we can expect certain features of our surfaces of constant phase difference to be uniquely characteristic of our model. The closest analogy is with wave propagation in nets of McCulloch–Pitts (1943) model neurons.

We start the development of a detailed theory of wave propagation by considering a regular two-dimensional array of close-packed hexagonal cells, as shown in Fig. 14. Let the pacemaker cell be a cell within the array away from its boundaries. Let it be on at the time t_0, signalling its ring of nearest neighbors. Suppose, for simplicity, that the time delay

$$\Delta t = \frac{\Delta\phi}{2\pi} T$$

between signal reception and signal generation within a given cell exceeds

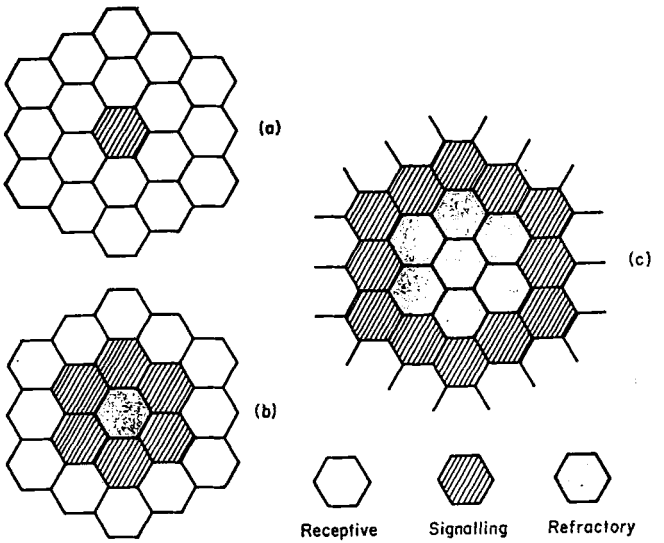

FIG. 14. Wave propagation in a regular two-dimensional array of close-packed hexagonal cells. The dominant cell is the central one. It is on at time $t = t_0$ in (a) and therefore signalling its ring of nearest neighbors. At time $t = t_0+\Delta t$, the central cell is off and in the refractory condition, as shown in (b). The ring of nearest neighbors of the dominant cell is signalling the next outermost ring. Note that the cells in the second ring are signalled alternately by one and by two cells of the first ring. This has no influence on the configuration shown in (c) at $t = t_0 + 2\Delta t$, where the second ring is on, signalling the next ring and the cells interior to the second ring are refractory.

the duration τ of the signal. Here $\Delta\phi$ is the phase shift of the event between adjacent cells introduced in the previous section, and T is the period. Then at $t_0+\Delta t$, the pacemaker will be refractory and the first ring of cells will be on and signalling the second ring. Similarly at $t_0-2\Delta t$, the pacemaker and the first ring will be refractory with the second ring on and signalling the third ring. Thus a circular wave front propagates radially outward from the pacemaker, with a mean propagation velocity

$$v = \xi \frac{d}{\Delta t} = \xi \frac{2\pi}{\Delta\phi} \frac{d}{T}, \tag{A.1}$$

where d is the cell diameter given by the requirement that $\pi d^2/4$ is the area of a cell. The factor $\xi \lesssim 1$ arises from the fact that we are dealing with a discrete structure instead of a continuum and is approximately 0·9 for the hexagonal array of Fig. 14. The cells on the wave front are on, those ahead are off and receptive, and those behind are off and refractory. The circular wave front becomes a sphere in three dimensions; all else remains the same.

The factor ξ depends on the degree of irregularity in the packing of the cells, on the amount of interstitial space, and on other geometric details. Analysis of propagation in, e.g. close-packed arrays of spheres in the face-centered-cubic structure suggest it to be close to 0·9 for that structure also. It is therefore sufficiently accurate for our purposes simply to set ξ equal to unity.

We now develop a Huyghen's construction for the propagation of wave fronts. Consider a wave front of "on" cells in a tissue as in Fig. 15. Each

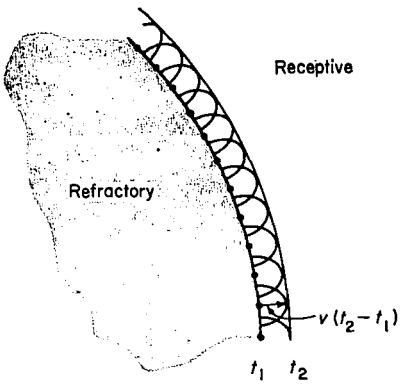

FIG. 15. Huyghen's construction for the propagation of wave fronts. Each cell on the wave front at time t_1 may be regarded as a pacemaker for the receptive region ahead of the wave front. During the interval $t_2 - t_1$, it would have caused all the cells in the hemisphere of radius $v(t_2 - t_1)$ centered on it to turn on. The overlap of spheres centered on the different cells on the wave front is irrelevant, multiple signalling not affecting the response. Therefore, the wave front at time t_2 is the locus of the extrema of the hemisphere, i.e. the surface tangent to the hemispheres. The second wave front is displaced from the first locally by a distance $v(t_2 - t_1)$ along the normal to the first.

cell on the wave front would by itself cause the turning on of all cells ahead of the wave front within a hemisphere of radius $v\delta t$ centered on it during a time interval δt. Let us draw such hemispheres about each cell on the wave front. The overlap of the spheres is irrelevant. A cell is turned on by the first hemisphere intersecting it; all later signals are of no consequence. Therefore, the wave front, being the locus of all cells that have just reached the on state, advances during δt to the locus of the extrema of the hemispheres and consists of those cells through which only one hemisphere passes; it is the surface tangent to all the hemispheres. The wave front advances along rays which are the trajectories of the normals to the wave front with a velocity v given by equation (A.1).

The geometric features of wave propagation established with the aid of

the Huyghen's construction can readily be re-expressed in terms of a differential equation for the surfaces of constant phase. Let

$$\Phi(\mathbf{r}, t) = 0 \tag{A.2}$$

be the equation for the surface coinciding with the wave front at $t = 0$. After a time interval δt, the points $\mathbf{r}+\delta\mathbf{r}$ will be on the wave front, yielding

$$\nabla\Phi\cdot\delta r - \frac{\partial\Phi}{\partial t}\,\delta t = 0 \tag{A.3a}$$

or

$$|\nabla\Phi|\,\delta n - \frac{\partial\Phi}{\partial t}\,\delta t = 0, \tag{A.3b}$$

where δn is the displacement of the wave front along its normal. From the Huyghen's construction, we know that

$$\frac{\partial n}{\delta t} = v, \tag{A.4}$$

yielding

$$\frac{\partial\Phi}{\partial t} = v|\nabla\Phi| \tag{A.5}$$

as the partial differential equation governing Φ. We are interested in the steadily propagating wave for which the separated solution

$$\Phi(\mathbf{r}, t) = \omega t + s(\mathbf{r}) \tag{A.6}$$

is appropriate, where ω is a constant. This gives

$$(\nabla s)^2 = \frac{\omega^2}{v^2} \tag{A.7}$$

for the equation governing s. From equation (A.2) we have

$$s(\mathbf{r}) = -\omega t(\mathbf{r}), \tag{A.8}$$

giving

$$(\nabla t)^2 = \frac{1}{v^2} \tag{A.9}$$

as the equation governing the time of propagation $t(\mathbf{r})$ from the pacemaker where $t = 0$ to the position $\mathbf{r}$ in the tissue.

Equation (A.9) is essentially the same as the eikonal equation of geometric optics. It applies to inhomogeneous tissues in which the cell size or the phase shift $\Delta\phi$ and hence the wave velocity v varies with position as well as to homogeneous tissues with constant v. It can be solved uniquely once boundary conditions are specified. Let F be the surface separating the pacemaker

region from the rest of the tissue. If there is only one pacemaker cell, F reduces to a point. The boundary condition on F is that

$$t(\mathbf{r}) = t_0 \text{ on } F, \tag{A.10}$$

where t_0 may be taken as zero for the S-event and $(1/2\pi)\phi_0 T$ for the P-event. For the R-event F is the surface where ϕ_{PS} equals ϕ_M and t_0 is correspondingly $(1/2\pi)\phi_M T$. In the latter case propagation proceeds away from F in both senses, in the former cases only outward from the pacemaker. The remaining boundary conditions relate to the behavior of the wave fronts at the bounding surfaces of the tissue outside the pacemaker region. This behavior can be established with the aid of the Huyghen's construction, but we postpone doing so until after we have derived from (A.9) the equation governing the positional information expressed as the PS phase difference ϕ_{PS}.

The relation between the time of arrival t and the phase of arrival ϕ of the wave front at the position $\mathbf{r}$ is

$$\phi = \frac{2\pi}{T} t, \tag{A.11}$$

which yields, when combined with (A.1) and (A.9)

$$(\nabla\phi)^2 = \left(\frac{\Delta\phi}{\xi d}\right)^2. \tag{A.12}$$

Equation (A.12) simply states that the derivative of the phase along the normal to the wave front is the phase shift between neighboring cells divided by the mean distance between them along the wave normal, an obvious and natural result which could have been arrived at directly. Equation (A.12) holds separately for both the P- and S-waves. However, because ϕ_P and ϕ_S both increase along the wave normal, because $\Delta\phi_P$ and $\Delta\phi_S$ are both positive and because $\Delta\phi_P > \Delta\phi_S$, we can take the square root of both sides of (A.12) for P and for S, subtract the results to give an equation for ϕ_{PS}, and square both sides of that to get

$$(\nabla\phi_{PS})^2 = \left(\frac{\Delta\phi_{PS}}{\xi d}\right)^2 \tag{A.13}$$

as the differential equation governing the positional information is developing tissue. Equation (A.13) is to be solved subject to the boundary condition that

$$\phi_{PS} = \phi_0 \text{ on } F. \tag{A.14}$$

We now establish the remaining boundary conditions on ϕ_{PS}.

Because of the profound influence of the refractory period, we must distinguish carefully between waves propagating into and away from the boundary. Let $\hat{n}_\phi$ be the unit vector normal to the wave front, i.e. the surface of constant ϕ, in the direction of propagation. Let $\hat{n}_B$ be the unit vector normal to the boundary surface of the tissue in the outward direction. We begin our analysis with the simplest case of plane waves propagating into, along, or away from a plane boundary as shown in Fig. 16. A wave is propagating into the boundary surface when

$$\hat{n}_\phi \cdot \hat{n}_B > 0. \tag{A.15}$$

The results of the corresponding Huyghen's construction are given in Fig. 16(a). One sees there that for propagation into the boundary B, the

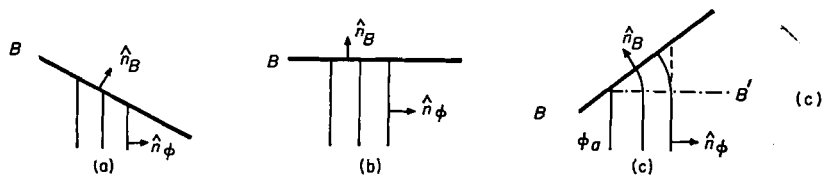

FIG. 16. Surfaces of constant phase in the vicinity of the tissue boundary. $\hat{n}_B$ is the outward unit normal of the boundary and $\hat{n}_\phi$ that of the surfaces of constant phase. The region of tissue shown is small enough for the boundary and wave fronts to be approximated by planes.

(a) Waves propagating into the boundary, $\hat{n}_\phi \cdot \hat{n}_B > 0$. (b) Waves propagating along the boundary, $\hat{n}_\phi \cdot \hat{n}_B = 0$. (c) Waves propagating away from the boundary, $\hat{n}_\phi \cdot \hat{n}_B < 0$.

The initial condition is that the tissue is refractory behind the surface of constant phase ϕ_a and sensitive ahead. The dashed lines are the analytic continuation of the surface ϕ_a to larger values of ϕ; the solid lines are the actual wave fronts according to the Huyghens construction. These consist of the continuations of ϕ_a inside the "illuminated" region bounded by B', joined smoothly on B' to circular cylindrical pieces lying between B and B' with an apparent source at the intersection of ϕ_a and B. These latter join B at right angles.

latter acts as a perfect absorber because of the refractory period. For propagation into the boundary there is no influence of the boundary on the wave. This is true in general, irrespective of the geometry as long as (A.15) is satisfied. The surfaces of constant phase are unaffected in shape by the boundary; they are merely bounded by it. Suppose that $\phi(\mathbf{r}) = \phi_a$ is a surface of constant phase within the tissue for which $\hat{n}_\phi \cdot \hat{n}_B > 0$ everywhere on the boundary. Take $\phi(\mathbf{r}) = \phi_a$ as a boundary condition for all $\phi(\mathbf{r}) > \phi_a$, and solve (A.12) ignoring the boundary. If one finds that $\hat{n}_\phi \cdot \hat{n}_B > 0$ everywhere on the boundary for all the $\phi(\mathbf{r})$ so determined, then one has obtained a unique solution equation (A.12). The argument carries over to surfaces of constant phase difference so that one obtains in this way a unique solution to (A.13). One simply integrates (A.13) outward from some initial surface

$\phi_{PS}(\mathbf{r}) = \phi_a$, ignoring the boundaries, and checks afterwards to see that the waves are everywhere propagating into the boundaries.

Similarly, as shown in Fig. 16(b), one finds that propagation along the boundary, $\hat{n}_\phi \cdot \hat{n}_B = 0$, does not modify the geometry of the surfaces of constant phase, provided the boundary is a plane. Modification of the surfaces of constant phase by the boundary occurs only in the case of propagation away from the boundary, $\hat{n}_\phi \cdot \hat{n}_B < 0$, as shown in Fig. 16(c). There we have employed an initial condition that the tissue is refractory behind a plane of constant phase ϕ_a, for which $\hat{n}_\phi \cdot \hat{n}_B < 0$, and sensitive ahead. If we were dealing with geometric optics, the plane of constant phase would propagate unmodified inside the "illuminated" region bounded by a plane B' normal to the wave fronts and intersecting ϕ_a in the latter's intersection with B. The region between B and B' would be "dark" for geometrical optics. Here, however, the Huyghen's construction shows the existence of cylindrical wave fronts centered on the intersection of ϕ_a and B as an apparent source, meeting B at right angles (i.e. satisfying the boundary conditions $\hat{n}_\phi \cdot \hat{n}_B = 0$ on B), and joining smoothly with the planes of constant phase across B.

The plane surfaces and plane boundary shown in Fig. 16 may be regarded as approximately correct in actual tissues for a sufficiently small region of the tissue. The surfaces of constant phase can in general be expected to be convex in the direction of propagation; this has no significant effect on the results presented in Fig. 16. The effects of curvature of the boundary are depicted in Fig. 17. Propagation into B, Fig. 16(a), is unaffected when the boundary is convex outward and also when it is concave as long as (A.15) is valid, as shown in Fig. 17(a) and (b). For propagation away from the boundary, curvature of B does cause the minor modifications of Fig. 16(c) shown in Fig. 17(c) and (d). The "illuminated" region inside B' is unchanged, but the "dark" region changes according to the sign of the curvature of B. For B convex, Fig. 17(d), the cylindrical wave fronts of Fig. 16(c) are intersected by B before the tangent plane T is reached so that the planes of constant phase are not perpendicular to B; otherwise there is no change. For B concave, Fig. 17(c), the cylindrical wave fronts are unchanged up to T where they arrive at right angles to T and join smoothly onto segments of the wave fronts which come in perpendicularly to B.

Convex or concave curvature of the surface B shown as plane in Fig. 16(b) results in a distinct modification of the surfaces of constant phase, as shown in Fig. 17(e) and (f). We suppose that initially the tissue is refractory behind a plane of constant phase ϕ_a and sensitive ahead, as in Figs 16(c) and 17(c) and (d). We suppose further that the analytic continuation of ϕ_a, $\phi_c > \phi_a$, is normal to B, as shown in Fig. 17(e) and (f). For the convex boundary, Fig. 17(e), a set of wave fronts between ϕ_a and ϕ_b have an "illuminated" and

a "dark" region exactly as in Fig. 17(c). Beyond ϕ_b, this case becomes identical to that of Fig. 17(a). For the concave boundary, Fig. 17(e), the situation is that of Fig. 17(b) for ϕ between ϕ_a and ϕ_c. The extremum of the boundary casts a "shadow", however, for ϕ greater than ϕ_c, and the situation there becomes identical to that of Fig. 17(d), but without the region between T and B'.

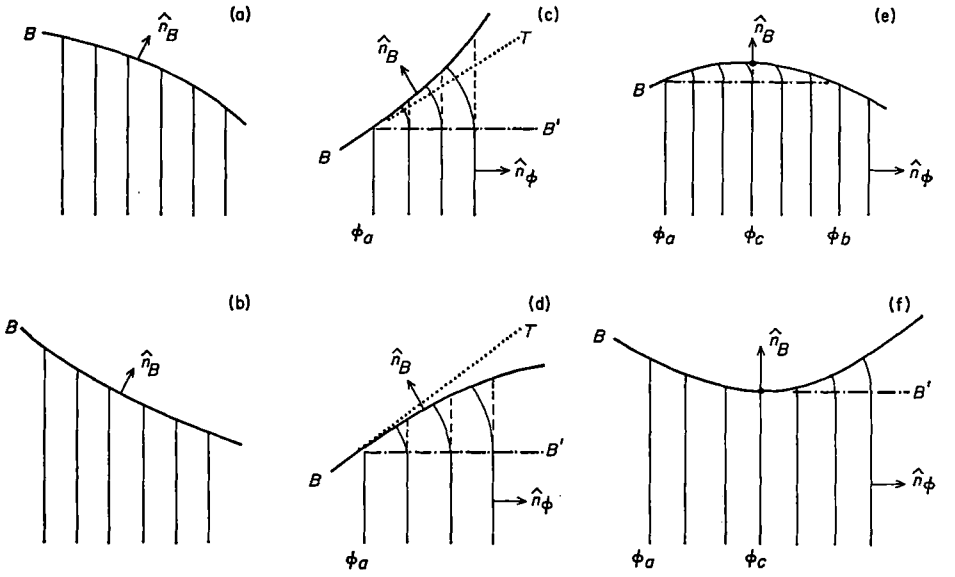

FIG. 17. Effect of boundary curvature on surfaces of constant phase near the tissue boundary.

(a) Propagation into a convex boundary. (b) Propagation into a concave boundary. (c) Propagation away from a concave boundary. T is the tangent plane to B at ϕ_a. (d) Propagation away from a convex boundary. (e) Propagation along a convex boundary. (f) Propagation along a concave boundary.

We see from the above analysis that the boundary value problem is straightforward as long as the waves propagate into the boundary. When the waves can propagate away from the boundary, the solution is necessarily piecewise continuous. Recourse to the analogous situations of illuminated and unilluminated or shadowed regions in geometrical optics is helpful. In any particular case the qualitative features of the wave fronts are usually easy to extract, although some quantitative features may be difficult. It is useful to remember that the time of propagation between two wave fronts is the same for any corresponding pair of points, as is the distance separating the wave fronts (for uniform cell size). In the particular case of a point

pacemaker region F on the surface of an everywhere convex tissue of uniform cells, the wave fronts are all spherical.

To summarize then, in the present section we have established a Huyghen's construction for O wave fronts (Fig. 15), have derived their equation of propagation [e.g. equation (A.13)], have established the absorbing boundary condition when (A.15) is satisfied, and have described the more complex wave fronts obtained when it is not. This suffices to define completely the surfaces of constant phase within a tissue of any geometry and with any given spatial distribution of cell sizes. All qualitative and quantitative features of the positional information are therefore completely defined and calculable within our model including the relation between positional information and embryonic geometry, with only the phase shift $\Delta\phi$ (which enters as a scale factor) presently unknown.