Timers In Developing Systems

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Summary. Conditional methods are proposed for investigating the number and relationships of processes that are rate-limiting for the genesis of consecutive stages in a developmental sequence. These methods depend on the differential sensitivity of "timer" pathways to small changes in temperature and can be applied to any developmental sequence in which discrete stages can be reproducibly monitored with time. We have applied the methods to multicellular morphogenesis in the slime mold *Dictyostelium discoideum* and have obtained an unexpected tentative scheme for timer relationships. A minimum of six timers has been delineated, each specific for at least one morphological stage. The majority of these timers appear to be in parallel.

The regulation of timing in developing systems has been a relatively neglected subject. Although an enormous effort has been directed at deciphering the relationships of processes essential for the genesis of stages in developing systems (*1*), no formalized methods have been advanced for distinguishing and characterizing those processes that are rate-limiting or that specifically function as developmental "timers." It has been assumed that, since there is a temporal order to the observable stages of a developing system, the underlying processes that regulate timing must also be in sequence. Although this may be true for the regulation of the timing of stages in dependent pathways in systems as simple as the cell cycle, it may not be true for more complex developing systems. For instance, in *Dictyostelium* morphogenesis, evidence has been presented suggesting that the processes which are rate-limiting for aggregation are in parallel rather than in sequence with the processes which are rate-limiting for subsequent morphological stages (*2*).

In this article a very simple approach is presented for investigating the number and relationships of those processes specifically involved in the regulation of developmental timing. The approach exploits the differential sensitivities of these processes to small changes in temperature. Its empirical usefulness is explored through application to a specific case, that of *Dictyostelium* morphogenesis. However, the approach is applicable to any developmental sequence in which discrete stages can be reproducibly timed.

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Not All Essential Processes Are Timers

The method that I have developed depends on a very simple construct: The time to a developmental stage is a reflection of the time that it takes to complete the last or slowest of several processes essential for that stage. To make this point more concrete, let us assume that in order to generate stage B in a developing system, four components which are the end products of four parallel processes must accumulate to minimum concentrations. If components a, b, and c accumulate to sufficient levels early and component d accumulates later, then the pathway for the accumulation of component d will regulate the timing of stage B (Fig. 1). Therefore, the processes that compose the pathways leading to the accumulation of a, b, and c are all essential, but they are not rate-limiting or timing processes. In contrast, the process that leads to the accumulation of d is both essential and rate-limiting for the genesis of stage B. The important point is that not all essential processes regulate the time of occurrence of a developmental stage.

In the present context, a timer could be either a single reaction or a sequence of reactions (that is, a pathway). However, the method for analyzing timers can be developed more simply if it is assumed that a timer is a uniform process in operation throughout the time period for which it is rate-limiting.

Possible Models for the Regulation of Timing

There are three basic models for the regulation of the timing of stages in a developmental sequence (Fig. 2) (*2*); these are a single timer model and two quite distinct multiple timer models. In the case of a single timer, there is one common rate-limiting process for the entire sequence of developmental stages, and this timer continues to function throughout the entire sequence. For both of the multiple timer models, there is a rate-limiting process specific for each developmental stage. In the sequential model, the start of each successive rate-limiting process is regulated by the termination of the previous rate-limiting process. In the parallel model, this is not the case; rather, rate-limiting processes for successive stages start and stop independently of one another and therefore function in parallel with one another. More elaborate models can obviously be constructed by combining these three basic models in various ways. One of the virtues of the approach to be described is that appropriate application permits the dissection of such complexity in terms of the number and relationships of detectably different rate-limiting processes.

Approach for Distinguishing Between Timer Models

The approach for distinguishing between timer models is separated into two experimental methods: The main utility of the first is to help distinguish multiple from single timer models, whereas the main utility of the second is to help distinguish whether multiple timers function in sequence or in parallel.

The first method for distinguishing between multiple and single timers is based on the following simple construct. If the times of occurrence of two or more stages are regulated by a single rate-limiting process, a small change in an environmental parameter should affect the times of occurrence of these stages uniformly, while the times of occurrence of stages regulated by different rate-limiting processes may be affected differentially. Although I use temperature as the environmental variable in developing and applying the method, other environmental variables could be used. Whether a

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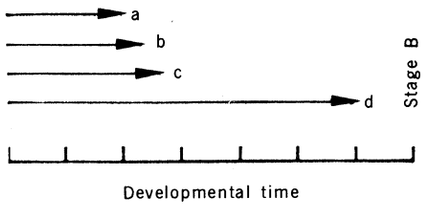
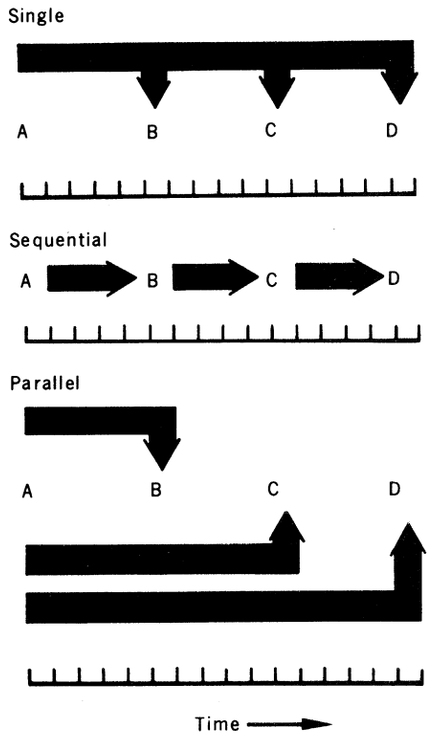


Fig. 1 (left). Hypothetical scheme for the genesis of a stage in a developing system. Four parallel processes, represented by four parallel arrows, produce four end products (a, b, c, and d), all essential for the genesis of stage B. The end point of each arrow represents the time it takes for the end product of each process to accumulate to the minimum concentration necessary for the genesis of stage B. In this scheme, the processes for the syntheses of components a, b, and c are essential but not rate-limiting; the process for the accumulation of component d is essential and rate-limiting since it is the last to be completed. Fig. 2 (right). Three basic models for the regulation of the timing of stages in a developmental sequence. Each horizontal bar represents an independent timer. The horizontal axis of each arrow reflects the progress of the timer. The vertical axis, or axes, points to the stage, or stages, regulated by the timer.



single timer model is applicable or whether it is excluded, leaving only a multiple timer model interpretation, can be tested experimentally by timing the stages of a developmental sequence at different temperatures within a very limited range. The test range should border the temperature that results in the maximum rate of development, and it should be in the direction of decreasing temperature (Fig. 3). The range should be as limited as possible to ensure that the relative order of the timing processes is maintained and that the temperature change affects rate-limiting processes in a relatively linear fashion (these points are discussed below). The times to the stages are then plotted as functions of temperature. Two indices are then calculated for each stage. First, the Δt, the actual difference between the times to a stage at the extremes of the temperature range, is calculated by the formula:

$$\Delta t = t_1 - t_2 \qquad (1)$$

where t_1 is the time to a stage at the lower temperature T_1, and t_2 is the time to a stage at the higher temperature T_2 (see Fig. 3 for a graphical description of T and t). Second, the $\%\Delta t$, the percent difference in times between the extremes of the temperature range, is calculated by the equation:

$$\%\Delta t = \frac{t_1 - t_2}{t_1} \times 100 \qquad (2)$$

The Δt's for different stages afford direct measures of the increases in time to stages with a decrease in temperature; the $\%\Delta t$'s also afford a test for uniformity since, if $\%\Delta t$'s are equal for two or more stages, then the effect of a decrease in temperature on their timing is uniform. The relations between the Δt's and the $\%\Delta t$'s for two or more stages are used to determine whether they are timed by single or multiple timer processes. In certain cases, the relations between Δt's and $\%\Delta t$'s help to distinguish between parallel and sequential timer models.

The measures Δt and $\%\Delta t$ can be either positive or negative, depending on whether a decrease in temperature increases or decreases the time to a particular stage. Our discussion will be limited to a very small temperature range in which the change is positive (Fig. 3) and in which the change in time throughout the temperature range is roughly linear.

In Fig. 4, hypothetical data for the possible effects of decreasing temperature on the timing of the sequential stages B and C of the developing sequence A to D are presented. In addition, the relations between the Δt's and $\%\Delta t$'s and the most likely, as well as the excluded, timer models are presented for each set of data. The cases presented in Fig. 4 will be dealt with as follows.

$\Delta t_B < \Delta t_C$; $\%\Delta t_B = \%\Delta t_C$. When a decrease in temperature uniformly affects increases in the times to stages B and C, the actual change in the time to B, (Δt_B), is less than the actual change in the time to C, (Δt_C); but the percentage changes in time for the two stages, $\%\Delta t_B$ and $\%\Delta t_C$, are equal (Fig. 4a). This is the only case in which a single timer model is applicable. Since a single timer would function continuously throughout the time period prior to the last stage regulated by it, the timer would be affected continuously by the decrease in temperature. Therefore, the increases in the times to stages regulated by the timer would be uniform, but the Δt for each stage would be greater in absolute value than the Δt for each previous stage. Multiple timer models are also applicable in this case. If parallel or sequential timing processes exhibited the same temperature sensitivities, then the effects on the times to the stages regulated by these processes would also be uniform, and the $\%\Delta t$'s would be equal.

$\Delta t_B < \Delta t_C$; $\%\Delta t_B < \%\Delta t_C$ or $\%\Delta t_B > \%\Delta t_C$. When a decrease in temperature affects a larger increase in the time to stage C than in the time to stage B in a nonuniform fashion, the Δt_B is less than the Δt_C; but the $\%\Delta t_B$ may be either smaller (Fig. 4b) or larger (Fig. 4c) than the $\%\Delta t_C$. In either case, because the effects on the timing of stages B and C are nonuniform, the single timer model is excluded. In these cases, no distinction can be made between parallel or sequential timer models. In either case, the rate-limiting processes for stages B and C exhibit different temperature sensitivities.

$\Delta t_B = \Delta t_C$; $\%\Delta t_B > \%\Delta t_C$. When a decrease in temperature affects equal changes in the times to the two stages B and C so that Δt_B is equal to Δt_C, the $\%\Delta t$ of the earlier stage, B, is greater than the $\%\Delta t$ of the later stage, C (Fig. 4d). In this case, the plots of developmental time as functions of temperature for stages B and C are parallel to each other. Since the $\%\Delta t$'s for the two stages are different, the effects of temperature on the timing of the two stages are nonuniform, excluding the single timer model. Both the sequential and parallel timer models are applicable. If the rate-limiting processes for B and C are sequential, then the one for B would be sensitive to the decrease in temperature since the interval between zero and B increases; but the one for C would be completely insensitive since the interval between B and C remains constant throughout the temperature range. If the rate-limiting processes for B and C are parallel, then the one for B would be slightly more sensitive than the one for C. The differences in sensitivity would coincidentally generate parallel lines.

$\Delta t_B > \Delta t_C$; $\%\Delta t_B > \%\Delta t_C$. When a decrease in temperature affects a larger increase in the time to stage B than in the

time to stage C, both the Δt_B and the $\%\Delta t_B$ are greater than the Δt_C, and the $\%\Delta t_C$, respectively (Fig. 4e). Since the effects of temperature on the timing of the two stages are not uniform, the single timer model is excluded. The parallel timer model is applicable. In this case, the rate-limiting process for stage B would be more sensitive to a decrease in temperature than the parallel rate-limiting process for stage C. The sequential timer model is applicable, but less likely, since it would require that the rate-limiting process for stage C increase in rate as the temperature decreases over the tested range.

In all nonparallel plots (Figs. 4, a, b, c, and e), there is the possibility that either an increase or decrease in temperature will cause a change in the order of stages. To exemplify this possibility, I have employed the relationship in Fig. 4e, in which $\Delta t_B > \Delta t_C$ and $\%\Delta t_B > \%\Delta t_C$. In this case (Fig. 4f), a decrease in temperature causes an increase in the time to stage B so that within the lower portion of the tested range, stage B occurs after stage C. In all such cases, only the parallel timer model is applicable; both single and sequential models can be excluded. These cases are unlikely in a developing sequence in which all transient stages are necessary and preparatory for following stages.

The preceding method of monitoring the times to developmental stages at varying temperatures within a limited range excludes or includes single timer models, but does not distinguish between parallel and sequential timer models in most cases. For this purpose, temperature shift experiments are used. If a system is allowed to develop to stage B at the high temperature of the test range and is then shifted to the low temperature of the range (Fig. 5), the time interval between B and C will be predictably different, depending on whether the rate-limiting pathway for C is in sequence with, or parallel to, the rate-limiting pathway for B. If the pathways are in sequence, then the interval time between B and C for a developing system shifted to the lower temperature at the time B occurs will be the same as the interval time between B and C for the system maintained at the lower temperature from time zero. If the processes are in parallel, then the interval between B and C after a shift to the lower temperature at the time B occurs will equal the time it takes for completion of the remainder of the pathway now functioning at the lower temperature and therefore at a lower rate. This predicted interval time can be obtained by first calculating the proportion of the pathway leading to stage C that was not completed during the period at the higher temperature, prior to the shift to the lower temperature. The proportion in this case is:

$$\frac{t(C_2) - t(B_2)}{t(C_2)} \qquad (3)$$

where $t(B_2)$ and $t(C_2)$ are the times to stages B and C, respectively, at the higher temperature (Fig. 5). By multiplying the time it takes for the genesis of stage C at the lower temperature, $t(C_1)$, by the proportion of the pathway not completed at the higher temperature, one obtains the predicted interval time, $t(BC)$, between stages B and C:

$$t(BC) = t(C_1)\left(\frac{t(C_2) - t(B_2)}{t(C_2)}\right) = t(C_1)\left(1 - \frac{t(B_2)}{t(C_2)}\right) \qquad (4)$$

This formula is predicated on the assumption that parallel rate-limiting processes are continuously in operation throughout the period prior to the stage for which they are rate-limiting.

The same distinction between parallel and sequential pathways can be made by a shift from low to high temperatures. If the rate-limiting pathways for stages B and C are in sequence, then a shift-up at B will result in an interval time between B and C which is equal to the interval time for the system maintained at the higher temperature from time zero. If the pathways for stages B and C are parallel, then a shift from low to high temperature at the time stage B occurs will result in an interval equal to the time it takes for completion of the remainder of the pathway to stage C now functioning at a higher temperature and therefore at a higher rate. This predicted interval time can be obtained by first calculating the proportion of the pathway leading to C that was not completed at the lower temperature

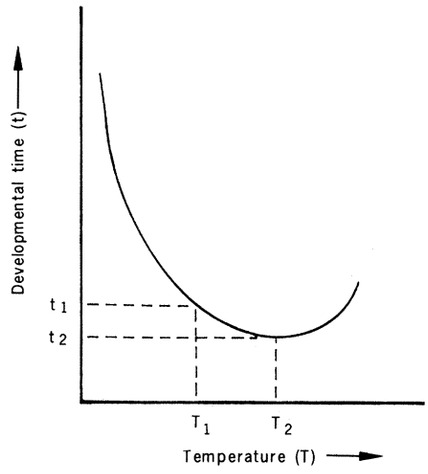


Fig. 3. Hypothetical temperature sensitivity plot for the timing of a developing system. T_1 and T_2 represent the lower and upper temperature limits, respectively, of the test range for the proposed method. t_1 and t_2 represent the respective developmental times at the lower and higher temperature limits.

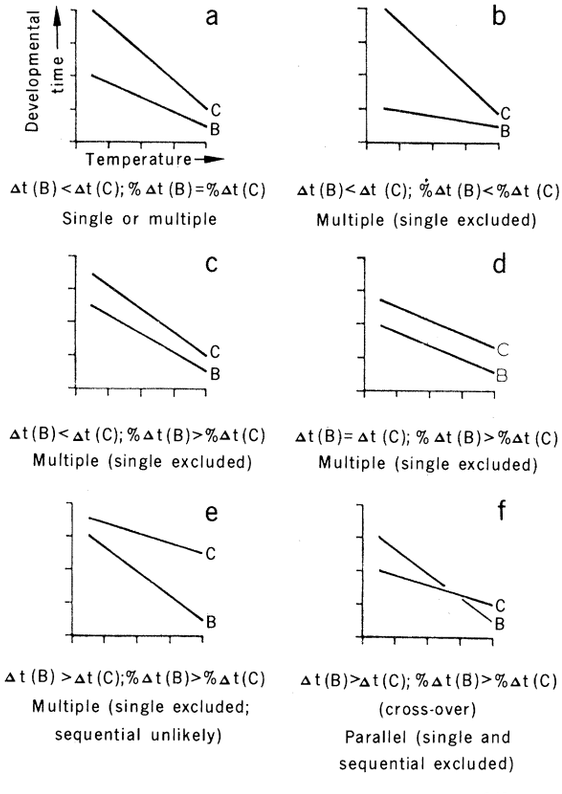


Fig. 4. Hypothetical data for the timing of sequential stages B and C in a limited temperature range. Δt and $\%\Delta t$ relationships between the two stages and the most likely as well as excluded timer model interpretations are presented for each set of data.

prior to the shift to the higher temperature. The proportion in this case is

$$\frac{t(C_1) - t(B_1)}{t(C_1)} \quad (5)$$

where $t(B_1)$ and $t(C_1)$ are the times to stages B and C, respectively, at the lower temperatures (Fig. 5). By multiplying the time it takes for the genesis of C at the higher temperature, $t(C_2)$, by the proportion of the pathway not completed at the lower temperature, the predicted interval time, $t(BC)$, between stages B and C is

$$t(BC) = t(C_2)\left(\frac{t(C_1) - t(B_1)}{t(C_1)}\right) = t(C_2)\left(1 - \frac{t(B_1)}{t(C_1)}\right) \quad (6)$$

To exemplify the usefulness of the shift experiment, the expected intervals are compared for sequential and parallel timer models after a shift at stage B from the lowest to highest and from the highest to lowest temperatures in the test range for the hypothetical data presented in Fig. 4e and replotted in Fig. 5. If the rate-limiting process for stage C is in sequence with the rate-limiting process for stage B, then a shift at stage B from the highest to lowest temperature in the tested range will result in an interval time between B and C of 0.5 arbitrary time units, and a shift from the lowest to highest temperature in the range will result in an interval time of 2.0 time units. However, if the pathways are in parallel, the interval times, calculated by the formulas for parallel pathways, will be 2.8 time units for the shift down and 0.36 time units for the shift up. The differences between predicted values for the two models are large and are dramatized by comparing the proportions of the shift down to shift up values for the two models. For the sequential model, the proportion is 0.25, and for the parallel model 7.8, a 30-fold difference.

The shift experiment will not distinguish between timer models when the effects of a decrease in temperature on two stages are uniform as in Fig. 4a. In this case, the predicted intervals for temperature shifts for single, parallel, and sequential timer models are identical.

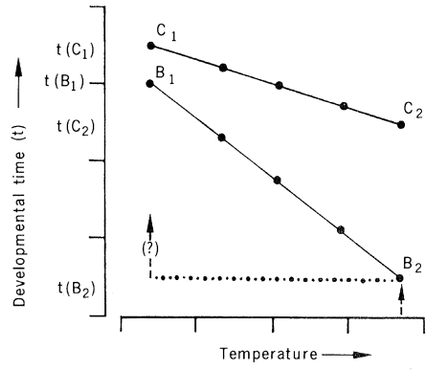


Fig. 5. Scheme for the temperature shift (down) experiment. The system is allowed to develop to stage B at the higher temperature, T_2, and then shifted to the lower temperature, T_1, of the test range. B_2 and C_2 represent the time points of stages B and C at the higher temperature for cultures maintained continuously at the higher temperature. B_1 and C_1 represent the time points of stages B and C at the lower temperature for cultures maintained continuously at the lower temperature. The dashed lines represent the program for the test culture. The dotted line represents the shift down.

Limitations and Assumptions

The methods formulated above ought to be useful for beginning to dissect the number and relationships of rate-limiting processes in developing systems, especially in the absence of other approaches. However, there are several limitations and simplifying assumptions which must be kept in mind. The more salient limitations are:

1) The number of rate-limiting pathways dissected by the proposed methods furnishes a minimum estimate, limited by the number of stages that are reproducibly monitored.

2) For the same reason, the proposed methods provide no information on the complexity of a single rate-limiting process. A "process" is distinguished by the capacity to separate it from other rate-limiting "processes" and may in turn be composed of several subprocesses.

3) The rate-limiting processes dissected by the proposed methods may not be the most mechanistically interesting in the genesis of a developmental stage. Many essential processes need not, and presumably are not, rate-limiting. In contrast, many rate-limiting processes may have no intrinsic "timer" identity other than that they are the slowest or last to be completed essential event under the conditions employed.

4) When a decrease in temperature affects two or more stages in a uniform fashion, a single timer model interpretation is viable, but so are multiple timer model interpretations. In this case, shift experiments can not distinguish between models.

Each of these limitations influences the resolving power of the proposed methods but does not impinge on the distinctions made between timer models. Three potentially troublesome, simplifying assumptions have been used in developing the proposed methods:

1) Each rate-limiting process is assumed to function continuously throughout the period for which it is rate-limiting, and it is assumed to do so at a relatively constant rate. For those rate-limiting processes in which the rate varies during this period, the formulation of the simple quantitative relationships presented above could become considerably more complex. For instance, a single process with a complex rate function could be interpreted to be composed of sequential timers.

2) It is assumed that the variation in

Table 1. The reproducibility of timing of monitored morphologies during *Dictyostelium* morphogenesis at 20°C. The figures represent the times in hours, at which 50 percent of the population on a developing pad (*2, 3*) exhibited the particular morphological stage.

Stage	Subclone 1 Exp. 1	Timing (hours)						Mean ± S.D.	S.D. (%)
		Subclone 2		Subclone 3					
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 3	Exp. 4		
Ripple	6.50	6.75	7.00	7.25	7.25	7.25	7.25	7.0 ± 0.30	4
Loose aggregate	9.25	8.75	8.75	9.50	9.75	9.75	9.50	9.3 ± 0.43	5
Tight aggregate	10.50	10.75	10.50	11.50	11.00	11.75	11.25	11.0 ± 0.49	4
Finger	13.50	13.00	12.50	13.25	13.50	13.75	13.75	13.3 ± 0.45	4
Early culminate 1	14.50	14.25	13.00	14.00	14.50	15.00	14.85	14.3 ± 0.65	5
Maxi-finger	16.50	15.75	14.50	15.75	16.00	16.50	16.25	15.9 ± 0.69	4
Early culminate 2	18.25	17.00	17.00	18.25	19.50	17.50	18.25	18.0 ± 0.88	5
Late culminate	21.25	20.75	20.00	20.75	21.75	21.00	21.25	21.0 ± 0.55	3
Fruiting body	28.50	31.00	27.50	29.25	29.00	29.50	29.00	29.1 ± 1.1	4

the environmental parameter—in this case temperature—does not result in a change in the identity of the rate-limiting process for each stage. This distinct possibility is the chief reason for using only a narrow range of variation near the optimum for the environmental parameter employed.

3) It is assumed that rate-limiting processes are affected in a roughly linear fashion by temperature shifts in the limited test range, and that all portions of a timer exhibit the same temperature sensitivity.

Rather than defend the usefulness of these simplifying assumptions at this point, it is prudent to apply the proposed methods to a real system, *Dictyostelium* morphogenesis. Certain of the results help to justify the usefulness of the assumptions as first approximations. In addition, methods are proposed for directly testing the third and most crucial of these assumptions.

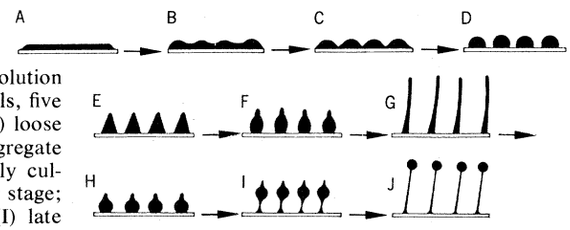


Fig. 6. The morphogenetic scheme for *Dictyostelium discoideum*, strain Ax3, clone RC3, developed on pads saturated with a buffered salts solution (*2, 3*). (A) Smooth carpet of cells, five cells deep; (B) ripple stage; (C) loose aggregate stage; (D) tight aggregate stage; (E) finger stage; (F) early culminate 1 stage; (G) maxi-finger stage; (H) early culminate 2 stage; (I) late culminate stage; (J) fruiting body stage.

Application of the Methods to *Dictyostelium* Morphogenesis

Morphogenesis in the cellular slime mold *Dictyostelium discoideum* is highly suitable for application of the proposed method. There are at least nine stages that can be monitored in a very short time period. In addition, the timing of each stage is highly reproducible under rigorously defined conditions (*2*).

When amoebas, in the log phase of growth, of the axenic strain of *Dictyostelium discoideum*, Ax3, clone RC3, are washed free of nutrient medium and are dispersed on a filter supported by two Millipore prefilters saturated with a buffered salts solution (*3*), they progress through a sequence of ordered stages leading to the final fruiting body (Fig. 6). At 20°C, the cells remain a smooth carpet for approximately 6 hours. By 7 hours, the carpet appears uneven or rough, and this stage is referred to as ripple (stage B). By 9 hours, the cell carpet separates into loose mounds of cells; this stage is referred to as loose aggregate (stage C). Each loose aggregate then constricts at its base, forming a near-perfect hemisphere by 11 hours; this stage is referred to as tight aggregate (stage D). Each tight aggregate forms a tip at its apex and elongates into a cone. By 13½ hours, the diameter at the base is approximately half the height; this stage is referred to as finger (stage E). Each multicellular unit then constricts at the base and elongates slightly at the apex forming a wine bottle shape by 14½ hours; this stage is referred to as early culminate 1 (stage F). Each multicellular unit then elongates radically into a long finger morphology by 16 hours; this stage is referred to as maxi-finger (stage G). Each unit then reverts to a squat wine bottle morphology by 17½ hours; this stage is referred to as early culminate 2 (stage H). Prespore cells then move up toward the apex of the unit as prestalk cells elongate to form a stalk attached to the substratum. By 21 hours, the spores have moved halfway up the stalk; the stalk is still rather thick and a nipple protrudes from the prespore mass; this stage is referred to as late culminate (stage I). By approximately 29 hours, each multicellular unit has formed a fruiting body (stage J), composed of a stalk supported by a basal disc and apexed by a spore cap.

A prerequisite for applying the proposed method to a developing system is that the timing of monitorable stages be reproducible between experiments. *Dictyostelium* morphogenesis fulfills this prerequisite. In Table 1, the times to the nine monitorable stages for three separate subclones of the major clone RC3 and for four separate experiments are presented. The standard deviations for all stages are low, never exceeding 5 percent of the means. Standard deviations are even lower within a subclone.

Since *Dictyostelium* morphogenesis is usually examined in the laboratory at 20° to 22°C (*3*) and since the maximum rate of development is attained at approximately 24° to 25°C, we selected the limited range of 18° to 24°C for an analysis of the effects of temperature on timing. The mean times and standard deviations for the nine described stages at 18°, 20°, 22°, and 24°C are presented in Table 2 for seven separate experiments. The mean times to each stage are also plotted as functions of temperature in Fig. 7.

By roughly examining the plots in Fig. 7, it is immediately evident that most of the stages of *Dictyostelium* morphogenesis are affected by a decrease in temperature in a nonuniform fashion and are therefore not regulated by a single timer. For instance, the time to stage B is relatively constant throughout the temperature range, but the times to all subsequent stages increase with decreasing temperature. The times to stages E, F, and G are very sensitive to a decrease in temperature and the temperature sensitivity plots are roughly parallel. The time to stage H is far less sensitive to a decrease in temperature than the stages immediately preceding it and the stages following it. However, to evaluate more critically the timer relationships between the different stages, the Δt's and $\%\Delta t$'s between the extreme temperatures 18° and 24°C of the tested range were calculated for each stage (Table 2). The relations between the Δt and $\%\Delta t$ of each stage and those of subsequent stages, as well as the timer models compatible with the data, are presented in Table 3.

1) *Ripple, loose aggregate, and tight*

Table 2. The time (in hours) to morphologies at four temperatures. The mean and standard deviation of seven sets of timing data involving three subclones and seven separate experiments are presented for each morphology at each temperature. The Δt and $\%\Delta t$ were calculated from the mean times to each stage at the extreme temperatures of the tested range 18° and 24°C.

Stage	Time to morphologies (hours)				Δt	$\%\Delta t$
	18°C	20°C	22°C	24°C		
Ripple	7.2 ± 0.27	7.0 ± 0.30	6.9 ± 0.37	7.2 ± 0.13	0	0
Loose aggregate	10.2 ± 0.44	9.3 ± 0.43	8.9 ± 0.40	8.8 ± 0.43	1.4	13.7
Tight aggregate	12.3 ± 0.58	11.0 ± 0.49	10.3 ± 0.43	9.9 ± 0.14	2.4	19.5
Finger	14.8 ± 0.58	13.3 ± 0.45	12.2 ± 0.49	11.1 ± 0.25	3.7	25.0
Early culminate 1	15.9 ± 0.65	14.3 ± 0.65	13.1 ± 0.61	12.0 ± 0.50	3.9	24.5
Maxi-finger	17.5 ± 0.47	15.9 ± 0.69	14.4 ± 0.70	13.0 ± 0.67	4.5	25.7
Early culminate 2	18.9 ± 0.85	18.0 ± 0.88	17.3 ± 0.80	16.9 ± 0.66	2.0	10.6
Late culminate	23.3 ± 1.10	21.0 ± 0.55	19.8 ± 0.89	18.9 ± 0.75	4.4	18.9
Fruiting body	31.5 ± 1.20	29.1 ± 1.1	25.7 ± 0.55	23.8 ± 0.54	7.7	24.4

aggregate stages. The timing of the ripple stage (B) is insensitive to a decrease in temperature in the range tested (Fig. 7). The Δt and $\%\Delta t$ are both zero (Table 2). All the subsequent stages in the developmental program exhibit substantial Δt's and $\%\Delta t$'s by comparison. Therefore, the rate-limiting pathways for all subsequent stages are either in parallel or in sequence with that for the ripple stage (Table 3). This may seem unreasonable since one would expect the ripple stage to be part of a continuum in the aggregation process. However, other conditions have been found under which the timing of the ripple stage is shortened without affecting the normal timing of subsequent aggregation stages (*4*), an indication that these timing mechanisms are experimentally dissociable.

Both the Δt and $\%\Delta t$ of the loose aggregate stage (C) are less than those of the tight aggregate stage (Table 2). This case is represented in Fig. 4b and indicates that the rate-limiting processes for the separation of the cell carpet into independent loose mounds of cells and for the subsequent constriction of each mound into a near perfect hemisphere are either parallel or in sequence. Therefore, the separation into mounds and the subsequent constriction into a hemisphere may not represent a single process regulated by a single rate-limiting pathway.

A comparison of the Δt's and $\%\Delta t$'s of the three aggregation stages (ripple, loose aggregate, and tight aggregate) with the Δt's and $\%\Delta t$'s of all subsequent stages indicates that in most cases a common or single timer model is excluded (Table 3). In these cases, no distinction can be made between parallel or sequential timer models. However, other conditions have been found under which the timing of the three aggregation stages can be decreased without affecting the timing to all subsequent stages (*2*). These data exclude a sequential timer model and indicate that the timers for the aggregation stages are in parallel with the timers for subsequent stages.

2) *Finger, early culminate, and maxi-finger stages*. The timing of the finger (E) and the early culminate 1 (F) stages exhibit similar Δt's in the temperature range 18° to 24°C (Table 2). In addition, the $\%\Delta t$'s for the two stages are very close (Table 2). Since the temperature sensitivity plots appear to be in parallel (Fig. 7), they may reflect the case in Fig. 4d, in which the Δt's are equal and the $\%\Delta t$ for B is greater than the $\%\Delta t$ for C. In that the $\%\Delta t$'s are very close because of the very short interval between E and F at 18° and 24°C, a uniform effect reflected in Fig. 4a cannot be ruled out.

The Δt for maxi-finger (G) is slightly larger than that for early culminate 1. In contrast, the $\%\Delta t$'s for the two stages are very close to each other. This case is represented in Fig. 4a; it is the only case in which the single timer model is not ruled out. The Δt and $\%\Delta t$ of maxi-finger exhibit the same relationships to those for the finger stage. However, the very close interval times between these three stages makes it very difficult to assess differences in the $\%\Delta t$'s.

3) *Early culminate 2*. The timing of early culminate 2 (H) is far less sensitive to a change in temperature in the tested range than the timing of the stages immediately preceding it (Fig. 7); its Δt and $\%\Delta t$ are far less than those of the finger, early culminate 1, and maxi-finger stages (Table 2). This case is represented in Figs. 4e and 5 and indicates that the rate-limiting process for early culminate 2 is most likely in parallel with the processes for these preceding stages. In addition, the timing of stage H is far less sensitive to a change in temperature than the two stages following it; its Δt and $\%\Delta t$ are both less than those of these subsequent stages and represents the case in Fig. 4b, indicating that the rate-limiting pathway for H is either in sequence or in parallel with those for stages I and J.

4) *Late culminate and fruiting body*. The $\%\Delta t$ of the late culminate stage is very close to the $\%\Delta t$ of tight aggregate, and the $\%\Delta t$ of the fruiting body stage is very close to the $\%\Delta t$'s of the finger, early culminate 1, and maxi-finger stages. These relationships reflect the case (Fig. 4a) in which the single timer is not excluded. However, it is difficult to believe that stages so far apart as tight aggregate and late culminate, or finger and fruiting body, are regulated by a single timer.

Both the Δt and $\%\Delta t$ of the late-cul-

Table 3. Timer model interpretations for the stages of *Dictyostelium* morphogenesis. Cases under maintained temperature analysis refer to the Δt and $\%\Delta t$ relationships depicted in Fig. 4, a to e. Abbreviations: P, parallel; S, sequential.

Developmental stage		Case in Fig. 4	Timer model interpretations		
			Temperature analysis		Other conditional analyses*
Initial	Subsequent		Maintained	Shift	
Ripple (B)	C	b	P or S	P	
	D	b	P or S	P†	
	E	b	P or S		P
	F	b	P or S		P
	G	b	P or S		P
	H	b	P or S	P	P
	I	b	P or S		P
	J	b	P or S		P
Loose aggregate (C)	D	b	P or S	P	
	E	b	P or S		P
	F	b	P or S		P
	G	b	P or S		P
	H	c	P or S		P
	I	b	P or S		P
	J	b	P or S		P
Tight aggregate (D)	E	b	P or S	P	
	F	b	P or S		P
	G	b	P or S		P
	H	e	P		P
	I	a or c	P, S, or single		P
	J	b	P or S		P
Finger (E)	F	a or d	P, S, or single		
	G	a	P, S, or single		
	H	e	P		
	I	c	P or S		
	J	a	P, S, or single		
Early culminate 1 (F)	G	a	P, S, or single		
	H	e	P		
	I	c	P or S		
	J	a	P, S, or single		
Maxi-finger (G)	H	e	P	P	
	I	d	P or S		
	J	a	P, S, or single		
Early culminate 2 (H)	I	b	P or S	P	
	J	b	P or S		
Late culminate (I)	J	b	P or S	P	

*Timer model interpretations made from the effects of the growth phase (*2*) and stationary phase factors (*2*) on developmental timing. †A very tentative interpretation.

minate stage (I) are less than those of the final fruiting body stage (Table 2). This case is represented in Fig. 4b and indicates that the rate-limiting pathway for the late-culminate stage is either in parallel or in sequence with that of the final fruiting body stage (Table 3).

In order to distinguish between parallel and sequential timer models in cases where the single timer model could be excluded, the temperature shift experiment was used. Cultures were allowed to develop to an initial stage at 18° and 24°C; they were then transferred to 24° and 18°C, respectively, and the interval times between the initial and subsequent stage were observed. The predicted interval times after a shift for sequential and parallel timer relationships were calculated from the timing of parallel cultures that developed at 18° and 24°C without a temperature shift (as described above). In Table 4, data are presented for temperature shift experiments that were performed to determine the possible relationships between timers for (i) ripple and loose aggregate, (ii) loose aggregate and tight aggregate, (iii) tight aggregate and finger, (iv) early culminate 2 and late culminate, and (v) late culminate and fruiting body. In all these cases, the observed interval times following a shift up and a shift down were closer to the interval times predicted for parallel timers than to those for sequential timers (Table 4). This point is dramatized by comparing the observed and predicted values for the ratios of the shift down to shift up intervals. For the interval between ripple and loose aggregate, the predicted ratio for sequential timers was 1.7 and for parallel timers 0.89; the observed ratio was 0.73, similar to the predicted proportion for parallel timers, but less than half the predicted proportion for sequential timers. The same was true for the intervals between loose aggregate and tight aggregate and between tight aggregate and finger. For instance, in the latter case the predicted ratios in repeat experiments for sequential timers were 3.1 and 3.5, and for parallel timers 0.8 and 0.6; the observed ratios were 0.9 and 0.5, both very close to the predicted ratios for parallel timers but less than one-fourth the predicted ratios for sequential timers.

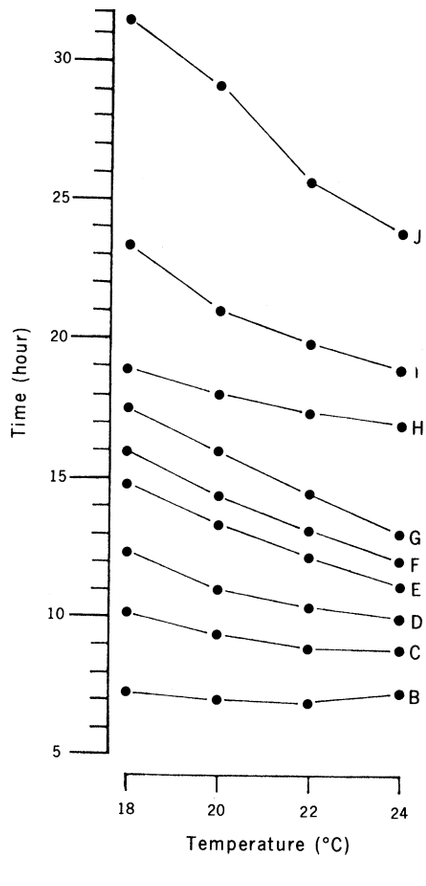
For the intervals between early culminate 2 and late culminate, and late culminate and fruiting body, the predicted ratios for parallel and sequential timer models were very close. Even so, the observed ratios were similar to the predicted ratios for parallel timers and dissimilar to the predicted ratios for sequential timers.

Maintained temperature analyses indicated that the timers for maxi-finger and early culminate 2 were parallel (Table 3). To retest this interpretation, the shift experiment was applied. The results again reinforced the parallel timer interpretation. The ratios of observed values was 2.3, close to the predicted value of 3.27 for parallel timers and dissimilar to the ratio of 0.41 predicted for sequential timers.

On the basis of the timer interpretations obtained by applying the methods formulated (Table 3), we have developed a tentative scheme for timer relationships in *Dictyostelium* morphogenesis (Fig. 8). A minimum of six timer processes have been delineated, each specific for at least one morphological stage (ripple, loose aggregate, tight aggregate, finger, early culminate 2, and late culminate). The majority of these timers appear to be in parallel. For four stages (finger, early culminate 1, maxi-finger, and fruiting body), distinctions could not be made between timer models since a decrease in temperature within the tested range affected uniform changes in their timing.

The observation that the majority of timers so far delineated are in parallel was surprising for at least two reasons. First, it was initially assumed that a timer represented no more than the last to be completed of several processes essential for the genesis of a particular stage (Fig. 1). Therefore, I expected to find both parallel and sequential timer relationships since it was assumed that essential processes, whether or not they were rate-limiting, would exhibit both parallel and sequential relationships. Second, because several of the morphological stages selected for monitoring appeared to represent transient points in a morphological continuum, I also expected that at least a few stages would be regulated by a single timer. The preponderance of parallel timer relationships has therefore stimulated a reassessment of the nature of timer processes, at least in the case of *Dictyostelium* morphogenesis.

It is possible that timer processes in *Dictyostelium* are far more specific than simply the slowest or last to be completed of several processes essential for the genesis of a particular stage. Key pathways may have evolved to specifically cue the genesis of stages and they may have evolved in parallel in order to ensure that other parallel processes which are essential but not rate-limiting were always completed prior to the gene-



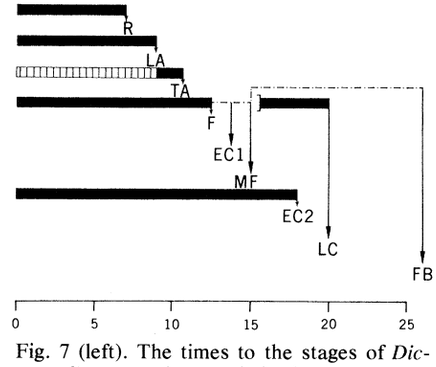


Fig. 7 (left). The times to the stages of *Dictyostelium* morphogenesis in the temperature range 18° to 24°C. Each point represents the mean of seven separate experiments. The standard deviations are presented in Table 2. The stages represented by the letters are presented in the legend to Fig. 6. Fig. 8 (right). A tentative scheme for timer relationships during *Dictyostelium* morphogenesis. Bars represent independent timers. The horizontal axes of each bar represents the progression of the timer. The dashed bar for *TA* represents a very tentative timer interpretation. The bracket at the beginning of the *LC* timer indicates that it is either in parallel or in sequence with the timer or timers for *F*, *EC1*, and *MF*. The dashed line indicates that no distinction could be made between timer models. Vertical arrows point to the morphological stages regulated by the timers. *R*, ripple; *LA*, loose aggregate; *TA*, tight aggregate; *F*, finger; *EC1*, early culminate 1; *MF*, maxi-finger; *EC2*, early culminate 2; *LC*, late culminate; *FB*, fruiting body.

Table 4. Distinguishing between sequential and parallel timer models for *Dictyostelium* morphogenesis by temperature shift experiments. Interval time between sequential stages for cultures maintained at 18° and 24°C were measured in each experiment. Predicted interval times for sequential and parallel timers were calculated by the methods formulated in the text for shifts from 24° to 18°C (shift down) and for shifts from 18° to 24°C (shift up). Each set of data represents an independently performed experiment. Best timer model interpretations are made for the timers of the stages bordering the interval.

Stages bordering interval	Interval time (hour)								Proportion of shift-down value to shift-up value			Best timer model interpretation
	Observed for cultures at		Predicted sequential		Predicted parallel		Observed		Predicted sequential	Predicted parallel	Observed	
	18°C	24°C	Shift-down	Shift-up	Shift-down	Shift-up	Shift-down	Shift-up				
Ripple, loose aggregate	3.4	2.0	3.4	2.0	2.5	2.8	2.4	3.3	1.7	0.89	0.73	P
	2.75	1.75	2.75	1.75	2.1	2.3	1.8	2.75	1.6	0.91	0.63	P
Loose aggregate, tight aggregate	2.5	1.0	2.5	1.0	1.5	1.7	1.0	2.0	2.5	0.89	0.50	P
	3.0	1.0	3.0	1.0	1.3	2.3	1.5	1.8	3.0	0.52	0.83	P
Ripple, tight aggregate	6.0	2.75	6.0	2.75	3.96	4.16	4.75	3.75	2.2	0.95	1.26	P*
Tight aggregate, finger	3.0	1.0	3.0	1.0	1.5	2.0	2.00	2.25	3.05	0.75	0.88	P
Maxi-finger, early culminate 1	1.3	3.0	1.3	3.2	3.6	1.1	3.0	1.3	0.41	3.27	2.30	P
Early culminate 2, late culminate	4.2	3.2	4.2	3.2	3.7	3.6	3.7	3.7	1.30	1.03	1.00	P
	3.7	2.8	3.7	2.8	3.1	3.3	2.9	3.4	1.32	0.94	0.85	P
Late culminate 2, fruiting body	7.0	4.25	7.0	4.25	5.9	5.0	6.0	5.0	1.65	1.18	1.2	P

*Very tentative interpretation.

sis of their respective stages. If timers had evolved in sequence, then a condition that selectively effected a decrease in the time of occurrence of an early stage would cause decreases in the cuing times for all subsequent stages. If this were the case, other parallel processes which were essential but not rate-limiting for the genesis of later stages may not have been completed when the genesis of the stage was cued; this could lead to the genesis of abnormal morphologies. In contrast, if timers evolved in parallel, then a decrease in the time of occurrence of an early stage would not affect the cuing times for subsequent stages, thus ensuring that other parallel processes which were essential but not rate-limiting would have been completed when genesis of the stage was cued. Whether other developmental systems comparable in complexity to *Dictyostelium* morphogenesis have evolved, parallel timing processes remains an open and testable question.

A Method For Testing Assumptions

The conditional methods formulated for examining the relationships of timers depend upon three assumptions, the most crucial being that all portions of a timer are affected by a change in temperature in a uniform fashion. This assumption can be tested by the following method. Cultures are shifted from the higher to the lower temperature of the test range at short intervals during the progress of the putative timer. Then, the difference between the time required to reach the stage regulated by the timer in a culture continuously at the lower temperature, $T(B_1)$, and the time to the stage after the temperature shift, $t(B_1AS)$, is plotted as a function of the time of the shift. The same analysis can be applied to shift-up experiments. The slopes of such plots reflect the temperature sensitivities of portions of the timer pathways, and changes in the slopes of such plots reflect timer complexity. In addition, the slopes of parallel portions of two or more timers can be compared. If the slopes of parallel portions of different timers are the same, the possibility of common timer portions with branch points cannot be excluded. This method is now being applied to the *Dictyostelium* system.

A Distinction Between Dependent Pathways and Timer Processes

A distinction can and must be made between dependent pathways and timer processes. In a dependent pathway, event B is a prerequisite to event C. A mutation or a condition which selectively blocks event B also results in the nonoccurrence of event C. A major objective in developmental genetics has been to order the actions of gene products along dependent pathways. By genetic and biochemical methods, multiple dependent pathways have been established for T4 phage assembly (*5*) and for the yeast cell cycle (*6*). In *Dictyostelium* morphogenesis, the increases in several developmentally associated enzyme activities depend upon a sequential set of morphogenetic events (*7*). Mutations that block morphogenesis at a particular stage result in the absence of enzyme activity increases that normally occur after that stage (*8*).

In most studies aimed at ordering the actions of gene products, a distinction is rarely made between "dependent" pathways and "timer" processes. This may be due to the inherent temporal order of dependent pathways. There has been an unexpressed assumption that because a dependent pathway consists of a temporally ordered sequence of essential processes, it must be responsible for temporally cueing the resulting developmental stage. This need not be the case. Dependent pathways can be essential for the genesis of a developmental stage without being rate-limiting (Fig. 1). A timer, however, is that essential pathway which serves as the temporal cue for the genesis of the stage under a particular set of conditions. The methods formulated do not examine all dependent pathways essential for the genesis of developmental stages. Rather, they examine exclusively the pathways that function as timers.

General Applicability of the Methods

Conditional methods for investigating the relationships between timing processes in developing systems have been developed. The methods can be applied to any system in which transient stages

can be reproducibly monitored with time. The methods are based upon three principle assumptions. (i) Rate-limiting processes are uniform and function at roughly constant rates throughout the periods for which they are rate-limiting; (ii) the identities of rate-limiting processes do not change with a shift in temperature within the small test range; (iii) rate-limiting processes are affected in a roughly linear fashion by temperature shifts within the limited test range. Although no direct proof is presented that these assumptions are correct in every case, the data obtained by applying the method to *Dictyostelium* demonstrate that they are useful first approximations. However, the methodology is oversimplifying. Although we have treated a timer as a uniform process, it is quite possible and probable that in many cases timer "processes" are not uniform and may even represent sequences of events that exhibit different temperature sensitivities. It is possible to expand the methodology to test this possibility by performing temperature shifts at short interval times during the period preceding each stage when the timer for that stage is progressing. Such an analysis would test whether all portions of a timer are affected in a uniform fashion by a temperature shift.

Although I have used temperature as the environmental variable in this study, other environmental conditions can be substituted. However, conditions other than temperature may not be as pervasive for all regions of a cell or for all regions of a multicellular system. Methods other than environmental perturbations can also be used to specifically investigate timers. These include the use of both metabolic inhibitors and mutations that differentially affect the timing of stages in a developing system. The conditional methods presented have so far been applied only to *Dictyostelium*. The results are interesting and provocative, but it must be kept in mind that, because of the assumptions and limitations of the methods, the scheme for timer relationships is tentative. In view of the lack of other methods, the ones I have developed should be valuable in initial attempts at understanding the regulation of timing in developing systems. Evaluation of the general usefulness of the approach must await the application of the methods to a wide variety of developing systems.

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Energy Choices for the Next 15 Years: A View from Europe

C. Pierre L.-Zaleski

Summary. A European perception of the world energy situation and its likely evolution during the next two decades is presented. French energy policy is then discussed as a possible set of consistent choices that can be made to deal with the energy situation in a regional context for the next 15 years.

It seems appropriate to introduce a discussion of energy choices by two remarks. These remarks are general, obvious, and were made many times before; however, they seem to me so important that I will take some of the reader's time to stress them again.

First, in dealing with energy planning, it is necessary to remember the long delays that in this field separate a decision from its effects on everyday life—in other words, the inertia of energy. The delays in energy production may be on the order of 10 to 20 years between the start of research and the technical demonstration of a process, 10 to 30 years between technical and commercial demonstrations of a new system, 10 years between the decision to build and the start-up of a power plant, and 10 years between the beginning of exploration for oil, coal, or uranium and operation of the mine or oil field.

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For energy consumption systems, there is also considerable inertia. For example, probably 60 percent of the buildings in France in the year 2000 will have been constructed before 1975. Therefore the new thermal insulation standard introduced in 1974 will apply to only 20 percent of buildings in 1985, 28 percent in 1990, and 40 percent in 2000. A new automobile model may be introduced after 5 years of study and design, sold for 5 to 10 years, and used for 10 years.

All this shows that an efficient energy policy should have continuity and persistence, should look far forward (20 to 25 years), and should not fluctuate with economic changes such as oil prices. Today's energy choices will have little or no effect on the real energy situation from now up to 1985 and perhaps longer. The energy situation in the year 2000 will, however, be almost fully defined by choices made in the next 15 years.

The second remark is about the specificity of each national situation and the simultaneous interdependence of local choices on each other and on the whole world economy. It is quite clear that demand, both quantitative and qualitative, and supply vary greatly from country to country and even from one region to another in a single country. Therefore each country and each economic region has to make its own choices on how to produce and how to organize the use of energy. It