been significantly retarded in their evolution (*1*). These results were more consistent with the clock hypothesis than with prevailing evolutionary theory, which assumed a simple relation between protein evolution and organismal evolution.

We have calculated an approximate standard error for the slope of the calibration line in Fig. 2 and constructed 95 percent confidence limits for the divergence times predicted from this equation. These limits must be regarded only as an approximate minimum estimate. To do this analysis, we assumed that the magnitude of the errors associated with each divergence time is the same for all values of immunological distance and that errors are normally distributed.

The error to which the calibration line in Fig. 2 is subject has several components: a paleontological error, an experimental error, and a stochastic or probabilistic error. The paleontological error is the result of the uncertainties in divergence time estimates, while the other two errors are independent of these estimates. The experimental error is the error in estimating how different the protein sequences are (by either direct sequencing or immunological procedures). The probabilistic error is an intrinsic feature of the clock. The clock is not metronomic (*1, 6*). The number of sequence changes that have occurred in a given protein, such as cytochrome c, in different species during a particular period of time follows a frequency distribution (*1, 11*). Several workers have attempted to measure this intrinsic error of the evolutionary clock for sequenced proteins (*6*) and for immunological comparisons of serum albumin (*12*). For sequenced proteins this error appears to be about twice as great as that expected for a probabilistic process like radioactive decay (*6*).

Even though the precision of protein clocks requires further definition, they appear precise enough to be extremely useful as tools for analyzing the dynamics of evolutionary processes during the past 100 million years or so. Protein clocks appear to have the potential for giving us a temporal view of evolutionary relations among all living species. By contrast, the fossil record is too poor to give us such a complete view. The vast majority of species do not have good fossil records. For this reason, it seems unlikely that paleontology will ever be able to estimate divergence times for more than a small fraction of living species that had common ancestors within the past 100 million years. With the approximate time depth between species provided by protein clocks, we can view the properties of those species from a time perspective. That is, we can calculate rates of evolutionary change for any property of the species being compared. Protein clocks are thus giving new perspectives on evolution at various levels of biological organization and generating new hypotheses concerning the biochemical basis of evolution at the supramolecular level (*1, 13*).

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Autonomous Timer in Malpighian Tubules

Friedman and Johnson (*1*) have suggested the existence of an autonomous biological timer in *Drosophila* Malpighian tubules which "assures synchrony between the appearance of urate oxidase activity in the tubules and emergence of the adult from the puparium." This conclusion is based on the demonstration that, in constant light, tubules transplanted from single, developmentally aged pupae expressed urate oxidase activity (in day-old hosts) at about the expected time of emergence for each pupa. The authors have also indicated that the circadian clock regulating emergence may have components in common with this Malpighian timer.

It is tempting to ascribe control of all manner of developmental events to a circadian oscillator. In fact, it would be pleasing if the temporal organization of the whole of development could be described in terms of circadian input. However, this viewpoint is not supported by the circadian literature. Pittendrigh and Skopik (*2*), working from earlier observations of Harker (*3*), have shown that at least two defined points in the development of three species of *Drosophila* (bristle pigmentation, yellow eye pigmentation) are not coupled to circadian oscillations. In addition, development in *Drosophila* clock mutants which display altered eclosion and activity profiles is seemingly normal (*4*).

It is also important to distinguish between two very different kinds of mechanisms which might both be called timers. A series of biochemical reactions in a defined developmental pathway could conceivably be called a developmental clock. This "clock" would probably not be temperature-compensated and, hence, rather inaccurate over a normal environmental range of temperatures. Eclosion and activity in *Drosophila*, on the other hand, are regulated on a circadian basis by a temperature-compensated multioscillator system (*5*). This second type of clock is inherently more accurate, since it is buffered against temperature changes.

The observation that Malpighian tubules autonomously express urate oxidase activity in a host supports the contention that the rate of development of

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enzyme activity is unaffected by transplantation of the tubules. A second observation made by Freidman and Johnson (*1*) is that this onset of increased activity coincides with the expected time of eclosion in constant conditions. Neither of these statements, however, warrants the conclusion that there is a timer which assures synchrony of the two developmental events in a normal environment: that is, in a light-dark cycle where emergence is a temporally regulated, "gated" phenomenon. If urate oxidase activity can be shown to be similarly gated in a light-dark cycle, then a hypothesis invoking clock control of enzyme activity might be acceptable.

Friedman and Johnson suggest the possibility that "components of the same clock regulating emergence also function in the Malpighian tubules to control the time of appearance of urate oxidase activity in the tubules." The eclosion clock is an accurate timer, but the "developmental clock" in Malpighian tubules has not been shown to be any more than a sequence of biochemical reactions (developmental events) leading to the expression of urate oxidase activity. If it is an autonomous timer analogous to the better studied clock that regulates activity and eclosion, then it should be possible to demonstrate resetting behavior and temperature compensation in addition to a gated expression of enzyme activity. Until this is achieved, it seems premature to speak of a "clock" that resides in the Malpighian tubules.

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Jackson (*1*), though he sees no fault with the data in our report (*2*), objects to the speculative remarks in the last paragraph. In our opinion Jackson's comments regarding our investigation can be condensed into the following two statements: (i) If there is only precedence in the literature for X, then one should not broach the possibility of Y. (ii) There are still important questions to be answered regarding the temporal control of urate oxidase activity in *Drosophila melanogaster*. We agree with the latter but not with the former.

We were aware (*2*) that Pittendrigh and Skopic (*3*) demonstrated that head eversion, yellow eye pigmentation, and ocellar bristle pigmentation in *Drosophila pseudoobscura*, *D. victoria*, and *D. melanogaster* are not coupled to the circadian oscillator which gates emergence and determines locomotor activity rhythms (*4*). Whether the onset of urate oxidase activity in the adult is coupled with emergence is an important question. The hypothesis that the appearance of urate oxidase activity in the adult and emergence behavior are coupled can be tested by phase-resetting experiments and transplant experiments (*5*). If there is absolute temporal coupling between the appearance of urate oxidase activity and emergence, another important inquiry must focus on the mechanism responsible. The contributions of humoral factors (*6*), emergence hormones (*7*), endogenous oscillators (*3*, *4*), and the developmental clock in the tubules (*2*) will have to be considered.

Jackson stated (*1*) that the " 'developmental clock' in Malpighian tubules has not been shown to be any more than a sequence of biochemical reactions (developmental events) leading to the expression of urate oxidase activity." We hasten to add that we have only really studied the behavior of the time-keeping mechanism in the Malpighian tubules and have yet to show directly that the timer in the tubules can be explained on the basis of a sequence of biochemical reactions, although we dogmatically and faithfully presume that all life forms and behaviors can be reduced to chemical reactions and interactions including temperature-compensated endogenous oscillators.

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Electropositive Potential Measured in Plant Protoplast

While being grateful to Racusen *et al.* (*1*) for citing us on the above subject, we would like to point out that our work referred to as unpublished in their report has appeared already (*2*).

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