Experiments Regarding the Problem of Differentiation in Multicellular Systems

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The temporal pattern of control by phytochrome of the enzyme lipoxygenase (EC 1.13.1.13) in the mustard seedling (Sinapis alba L.) is described. The biosynthetic system which produces lipoxygenase does not respond to phytochrome (Pr) up to 33.25 h after sowing (25 °C). By this time the control by Pr comes suddenly and fully into play. At 48 h after sowing the system escapes from the control by Pr. Neither the beginning nor the end of the period of control is influenced by phytochrome. Interpretation of the data requires a double action control mechanism. It is argued that such a mechanism is necessarily a characteristic of development in multicellular organism.

In this paper we want to deal from an experimental point of view with a major problem of development which has been repeatedly emphasized by developmental biologists (e.g., Waddington 1, 2 or Stern 3) but is commonly neglected by molecular biologists familiar with only microorganism: the problem of a double action control mechanism in development of multicellular systems. Although in the present paper the problem is not solved, it is at least explicitly stated on the level of regulation of enzyme synthesis. The problem must be visualized clearly if speculations about the molecular mechanisms that control the epigenesis of higher forms are to be consistent with the available facts.

Phytochrome-mediated morphogenesis in plant seedlings (Sinapis alba L.) is used as a model for a double action system 4. Standard far-red light 5 maintains in the mustard seedlings a relatively low 7 but virtually stationary 8 concentration of active phytochrome, Pr, over a considerable period of time. The ratio \( \frac{[Pr]}{[P_{tot}]} \) is in the order of 0.025, whereby Pr = physiologically active phytochrome, Pfr = physiologically inactive phytochrome, and Pfr = Pr + Pfr. The ratio is rapidly established, i.e., in the order of a minute, after the onset of far-red light. No Pfr can be detected in the dark grown seedling. The only detectable form of phytochrome present in the dark grown mustard seedling is Pfr.

The Problem on the Level of the Cell (Fig. 1)

It is generally agreed 9 that the genetic information in most complete cells of a complex multicellular organism is identical with that of every other cell. Within a given organism the tremendous diver-
sity of cell phenotypes appears to derive from the fact that each cell expresses only a limited amount of its full genetic potential and that different cell types express different portions of their genome.

In terms of this background we now analyse phytochrome-mediated photomorphogenesis. There are no data against the assumption that phytochrome is the same in all the organs and cells of a seedling in which it occurs. However, the different organs and tissues of a seedling respond differently to the formation of Pfr. We do not need to discuss the multiplicity of displays, we only draw the conclusion that the response which takes place depends on the specific state of differentiation of the cells and tissues. Pfr, can only trigger (or release) the response. It has nothing to do with the specificity of the response. This point of view is emphasized in Fig. 1. We look at segments of cross sections through the hypocotyl of mustard seedlings. Under the influence of far-red light, i.e., under the influence of Pfr, certain cells of the epidermis have formed hairs, and at the same time all the cells of the subepidermal layer (but no other cells) have formed anthocyanin. Obviously, the specificity of the cellular response (e.g., hair formation or anthocyanin synthesis) must depend on the specific state of reactivity (or competence in Waddington's terminology) of the cells and tissues at the moment when Pfr is formed in the seedling.

We have summarized the action of Pfr under the term secondary differentiation and we have called the process of differentiation which determines the cell's response to Pfr, primary differentiation. The term primary differentiation is probably in many instances equivalent to the term determination which is sometimes used by animal embryologists. Instead of secondary differentiation Waddington uses the term activation (by external stimuli like hormones).

Irrespective of terminology the important point is that we need a double action control mechanism in order to understand even the simple situation sketched in Fig. 1.

Any satisfactory theory of development of multicellular systems must account not only for the static situation illustrated in Fig. 1, but also for the changes in time. The problem is whether or not the temporal sequences of primary and secondary differentiation can be taken apart, not only logically but also experimentally. We postulate that the processes of Pfr-mediated secondary differentiation are fully programmed in the pattern of primary differentiation. We further postulate that the process of primary differentiation is independent of phytochrome. To check these postulates experimentally, we make use of the fact that synthesis of lipoxygenase is controlled by Pfr in the mustard seedling.

The Problem on the Level of Enzyme Synthesis: the Background

Synthesis of the enzyme lipoxygenase (EC 1.13.1.13) in the cotyledons of the mustard seedling is controlled by Pfr through a threshold mechanism. Standard far-red, e.g., immediately inhibits lipoxygenase synthesis and the repression can be maintained by continuous far-red over many hours. The following threshold concept is required to explain the control by Pfr of lipoxygenase synthesis: The repression of enzyme synthesis by Pfr, is a very rapid process after the threshold level is surpassed. Likewise, enzyme synthesis starts instantaneously and with full speed as soon as the Pfr level decreases below the threshold level. Thus Pfr rapidly inhibits synthesis of an enzyme and functions through an all or none control mechanism of high precision.

The experiments on the Pfr-mediated control of lipoxygenase synthesis were done between 36 and 48 h after sowing. The results and conclusions are valid for this period of time. The question is whether lipoxygenase synthesis responds to Pfr throughout the whole period of the seedling’s development (up to 84 h after sowing).

Materials and Methods

Standard techniques for photomorphogenetic research with mustard seedlings were used. The seedlings were grown continuously at 25 °C. A standard far-red source which maintains a Pfr/Ptot ratio in the order of 0.025 in the mustard seedling was used at an irradiance of 350 μW·cm⁻². The enzyme assay was described previously. The whole seedlings were used for extraction. Approximately 90% of the total extractable enzyme of the seedlings is localized in the cotyledons. The enzyme appeared homogeneous on

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disc electrophoresis. The term enzyme synthesis was justified previously. Standard errors of enzyme measurements are between 1.0 and 1.5 percent. This precision indicates that the cells of the mustard seedling involved in lipoxygenase production form a synchronized population. The lipoxygenase activity measured at 36 h after sowing is defined as 100 percent activity.

**Results**

The lipoxygenase kinetics in the dark is shown in Fig. 2. If one irradiates with standard far-red from the time of sowing there is no control of lipoxygenase synthesis up to 33.25 h. At this time the full repression of lipoxygenase synthesis by far-red (i.e., by a Pfr value above the threshold) comes suddenly into play, whereas at 48 h after sowing, the seedling suddenly and completely escapes from the control by Pfr. Enzym synthesis is resumed even under continuous far-red. Exactly the same temporal pattern is observed if the onset of continuous far-red is 24, 33.25 or 36 h after sowing. Fig. 3 shows clearly that the system which produces lipoxygenase always escapes from the control by Pfr at 48 h after sowing.

Spectrophotometric measurements of phytochrome performed with mustard cotyledons by Schäfer according to Marmé's methods indicate that neither at 33.25 h nor at 48 h are there any abrupt changes in the amount of detectable phytochrome. Furthermore, it can be concluded on the basis of the available data that before 33.25 h as well as after 48 h, the level of Pfr in the far-red is well above the threshold level. Therefore it is im-

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**Figures:**

Fig. 2. The increase of lipoxygenase in the mustard seedling in continuous dark and under continuous standard far-red light. The onset of far-red was at the time of sowing of the seeds (0 h). If necessary, we use in the following the abbreviations: dark = d, far-red = fr, continuous = cont.

Fig. 3. The increase of lipoxygenase in the mustard seedling in continuous dark and under the influence of standard far-red light. The onset of far-red was 24, 33.25 or 36 h after sowing of the seeds.

Fig. 4. The increase of lipoxygenase in the mustard seedling in continuous dark and under the influence of continuous standard far-red light. The onset of far-red was 33.25, 36 or 42 h after sowing of the seeds. Note that under all circumstances the system escapes from control by far-red at 48 h.
probable that the temporal pattern of response (no control up to 33.25 h, full control for 14.75 h and no control beyond 48 h) has anything to do with $P_{fr}$. This conclusion is supported by experimental results like the ones in Figs. 5 and 6. In spite of differing levels of $P_{fr}$, the system escapes from the control of $P_{fr}$ under all circumstances at 48 h after sowing. We conclude that the temporal pattern of response, summarized in Fig. 7, must be determined by changes in the system on which $P_{fr}$ acts and not by $P_{fr}$. In previous experiments$^{10}$ it was shown that Actinomycin D at 10 $\mu$g/ml completely and specifically inhibits the increase of lipoxygenase activity. Furthermore, it was concluded$^{10}$ that enzym turnover does not occur during the 48 h experimentation period and that any increase in enzyme activity represents a corresponding synthesis$^{10}$. The half lives of the transcription intermediates are relatively short while that of the enzyme is long$^{10}$.

**General Discussion**

Fig. 7 summarizes the results of the present paper. The system which produces lipoxygenase does not respond to $P_{fr}$ up to 33.25 h after sowing. At this time the control by $P_{fr}$ comes suddenly and fully into play. At 48 h after sowing the system completely escapes from the control by $P_{fr}$.

We conclude: firstly, the repressive action of $P_{fr}$ on lipoxygenase synthesis is obviously a function of primary differentiation. Secondly, the time course of primary differentiation is independent of $P_{fr}$. The latter conclusion is based on several facts: a) Neither the beginning nor the end of the period of control (from 33.25 h to 48 h after sowing) are to any detectable extent influenced by $P_{fr}$. b) After resumption of synthesis the lipoxygenase kinetics run into the dark kinetics rather abruptly. In other
words, as soon as the lipoxygenase kinetics reaches the kinetics in continuous dark, the control system acting in the dark takes over completely without any detectable after-effect of the light treatment.

The phenomenon which we have described is possibly of general occurrence. It has been recognized recently, at least in principle, in connection with the pattern of enzyme synthesis throughout the cell cycle of animal cells. An example is the induction of tyrosine aminotransferase by steroids which has been studied in synchronized cultures of HTC cells, an established line of rat hepatoma cells. An example is the induction of tyrosine aminotransferase by steroids which has been studied in synchronized cultures of HTC cells, an established line of rat hepatoma cells. Although the enzyme can be synthesized in all phases of the HTC cell cycle, it can only be induced by the steroids during certain periods, namely, in the latter two-thirds of the interval between mitosis and the onset of DNA synthesis and during DNA synthesis. During the period between DNA synthesis and mitosis, mitosis, and the first several hours of the interval between mitosis and DNA synthesis, synthesis of tyrosine aminotransferase is not influenced by the presence of the hormone. Tomkins and associates conclude that “during the noninducible phases of the cell-generation cycle, transcription of the tyrosine aminotransferase gene is repressed by a process insensitive to the steroid, and that this repression is lifted during the inducible periods of the cycle”.

This is another description for the hormone independent regulatory process we call primary differentiation.

We cannot explain yet primary differentiation in terms of molecular biology. The causalities of primary differentiation are unknown not only in plants but also in animals. But we can by now at least clearly describe the problem (Fig. 1).

There are models which might be useful in the further theoretical approach to the problem, e.g. the model elaborated by Tomkins and associates or the model proposed by Britten and Davidson and critically evaluated by Waddington. It is hoped that the clear recognition of the problem of primary and secondary differentiation on the level of enzyme synthesis will open the way towards an adequate theoretical biology of development and eventually lead to a molecular model of development in multicellular systems which is consistent with the facts of epigenesis.

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