MOLECULAR FACETS OF MITOTIC REGULATION, II. FACTORS UNDERLYING THE REMOVAL OF THYMIDINE KINASE[*,†]

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In the preceding article,[1] evidence was presented indicating that the brief and transient appearance of thymidine kinase during the mitotic cycle of microspores represented a *de novo* synthesis of at least part of the enzyme protein. The process was shown to have all the earmarks of induced enzyme syntheses in bacteria[2] except for the identity of the inducer. The nature of the latter remains unknown as does its site of origin. The fact that a transient pool of deoxyribosides forms outside the cells immediately preceding the appearance of thymidine kinase in the microspores[3] might be interpreted as signifying that such deoxyribosides do provide the inducing substance. Studies on this point have yielded no conclusive evidence. Under conditions of anther culture it is possible to reverse the order of appearance of pool and enzyme by means of an inhibitor such as azaguanine; it is also possible to stabilize the deoxyriboside pool in the presence of ethionine while completely inhibiting the appearance of enzyme. The solution to this question would appear to lie in studies of isolated microspores, but such studies have not yet been attempted. Instead, a number of experiments were conducted with the aim of clarifying the conditions which lead to the disappearance of thymidine kinase activity.

Methods.—The procedures used in these studies are identical with those described in the preceding publication.[1]

Results.—Energy requirements for enzyme removal: Despite the strong evidence for the synthesis of enzyme protein there may well be some uneasiness about the interpretation because of the well-established demonstrations in bacteria that induced enzymes persist after removal of inducer and become diluted only as a consequence of cell proliferation.[2] The comparison, to be sure, is not entirely valid since the enzyme would fall under the category of "constitutive" in a population of microspores which were not developing in close synchrony. Experiments were nevertheless sought which might throw some light on the process underlying the disappearance of enzyme. In this connection some early studies of Spiegelman and Reiner[4] on galactozymase induction in yeast proved to be pertinent. They had observed that induced galactozymase disappeared when galactose was removed from the medium, and that such disappearance was not a consequence of dilution due to cell proliferation. Moreover, they found that enzyme disappearance could be halted if the cells were deprived of their aerobic energy supply. In the light of these studies experiments were set up to determine whether the normal disappearance of enzyme could be halted by subjecting microspores to similar conditions. Accordingly, cultured anthers were exposed to dinitrophenol or an atmosphere of nitrogen at a time when they were expected to have formed enzyme. The transient nature of the enzyme made it advisable to favor earliness of exposure because of the possibility that, once the disappearance of enzyme began, it could not be halted. The results of these experiments are illustrated in Figure 1. In all cases (12
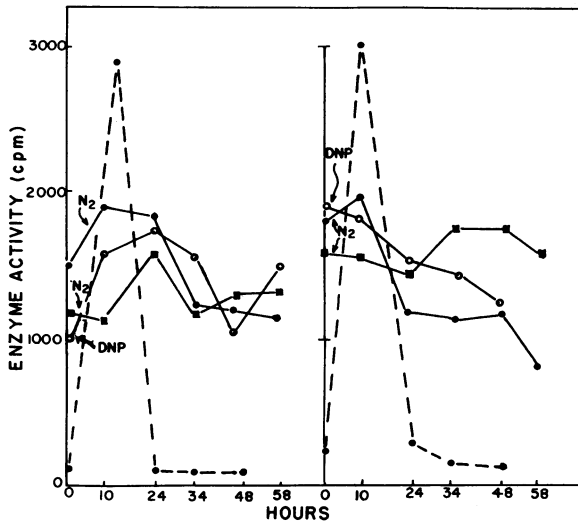
FIG. 1.—Inhibition of thymidine kinase disappearance by blockage of respiration. Each point represents the activity of microspores from $1/2$ anther. Dotted lines are control runs. Treatments were begun at the time the cultured anthers were expected to have approached the peak of enzyme activity,[1] and at that time one anther was removed from each culture tube for microspore isolation. Subsequent samples were taken at the intervals indicated. In the case of nitrogen treatment the culture tubes were evacuated 4 times and filled with nitrogen gas. In the case of dinitrophenol 600 μg of the reagent were added to 0.2 ml culture medium 2 days prior to the expected peak in order to allow for lag in penetration.

such series were tested), the behavior of thymidine kinase paralleled that of galactozymase. An extended duration of enzyme activity such as that caused by anaerobiosis has not been observed in the fifty or so control runs thus far made. The simplest conclusion which may be drawn from these studies is that the regulated removal of enzyme is an energy-requiring process which cannot be fulfilled under anaerobic conditions.

Regulatory factors in enzyme synthesis: The process of enzyme removal, though energy-dependent, remains obscure and its regulation is therefore an open question. If removal of enzyme has to be induced in much the same way as its formation, then it should be possible to block that removal by the same reagents which inhibit enzyme formation. Attempts to disrupt the removal process by treating microspores with azaguanine, fluorouracil, or chloramphenicol at various times during the enzyme cycle were uniformly unsuccessful. A departure from the normally sharp periodicity was only observed in some of the experiments involving the reversal of azaguanine action by guanine. These results, although suggestive of a disturbance in the regulatory mechanism, could be attributed to derangements in the enzyme induction process. The problem of finding a means to derange the periodicity other than that of a general choking of metabolic processes by anaerobiosis remained.

Since Taylor *et al.*[5] had observed that breakages in chromosomes could be repaired to some extent after the normal completion of DNA synthesis, we considered the use of techniques leading to such breakage as a means of interfering with the regulatory process. An assortment of techniques was chosen: X irradiation, acridine orange, mitomycin C, and fluorodeoxyuridine. The original plan was to cause chromosome breakage at the time of DNA synthesis and then to look for a reappearance of thymidine kinase activity. This plan did not materialize because it was observed that, with the exception of fluorodeoxyuridine, all the techniques led to a disruption of regulation prior to the interval of DNA synthesis. The ineffectiveness of fluorodeoxyuridine was not surprising, since its untoward physiological effects would be expected to arise after the onset of DNA synthesis.

The results were nevertheless of interest because, as may be seen in Figure 2, fluorouracil was, like azaguanine, a sure inhibitor of enzyme formation, whereas the deoxyriboside form had no trace of inhibitory action.

The assembly of curves in Figure 2 have a single and striking feature in common: they are all examples of a breakdown in regulation with greater or lesser degrees
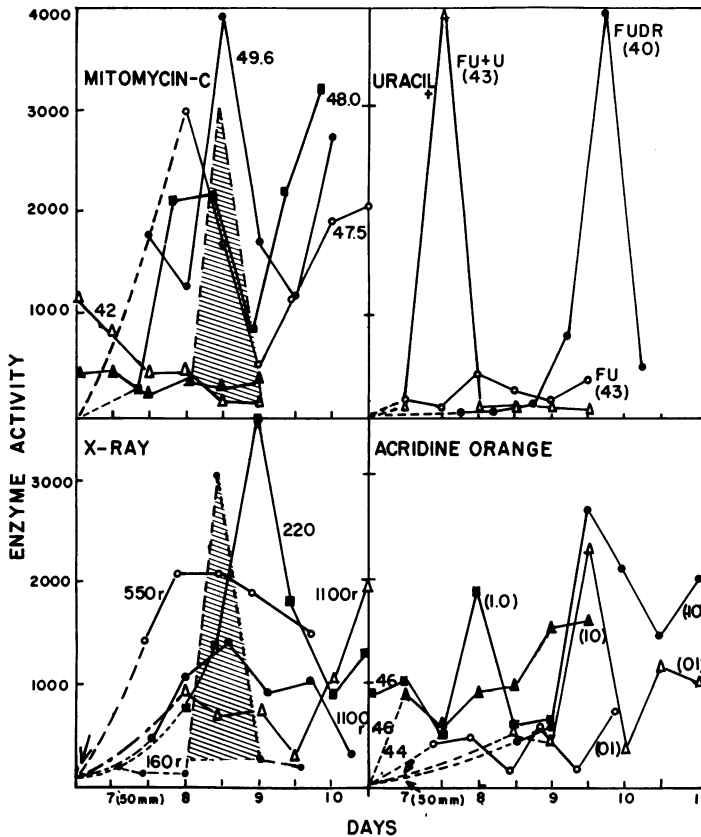

FIG. 2.—Disruption of enzyme regulation by agents affecting nucleic acids. Experimental arrangement as in previous figures. Arrows or, alternatively, equivalent bud length, indicate time of treatment. Enzyme activity expressed as cpm of H^3-thymidylic acid per microspores from $1/2$ anther. Each tube contained 0.2 ml Hoagland's solution. Mitomycin C was added to a concentration of 100 μg/ml; 5-fluorodeoxyuridine (FUDR), 0.267 mg/ml; 5-fluorouracil (Fu) 1 mg/ml; uracil (U) 2 mg/ml. Three different concentrations of acridine orange were used: 0.1 1.0, and 10 μg/ml; the pH of the medium was 6.3. Radiation doses in roentgens are shown adjacent to the corresponding curves. Anthers were irradiated at bud lengths between 46–50 mm; no distinctive differences were found between different lengths within this range. Shaded areas are typical for control runs. The shape of the area is nearly constant in controls although the location of the peak may vary within ±1 day.

of suppression of the enzyme-forming system. At one extreme the level of enzyme activity is close to that of inhibited cells; at the other, the level is at least as high as that found in control systems. Closer inspection of the curves permits a number of other pertinent observations. X irradiation at different times during the few days prior to enzyme appearance produced the most assorted pattern of effects. Among the 12 series run there appeared to be no relationship between the particular time at which the cells were irradiated and the pattern produced. There is an

obvious relationship between dose and enzyme level, but the temporal dispersion of enzyme activity is much the same over a broad level of doses. With relatively low levels of irradiation administered prior to enzyme synthesis, it is possible to distort the regulatory mechanism without measurably affecting the synthetic one.

Mitomycin C and acridine orange present a slightly different picture. Both of these reagents, if added at times when azaguanine and fluorouracil inhibit enzyme formation, have similar inhibitory effects, although acridine orange is a less effective inhibitor than the base analogues even at the highest concentrations used. Both these substances, however, have marked effects on regulation if added after the time when azaguanine is completely ineffective. The lags in peak activity, the twin peaks, and the persistence of intermediate values are entirely foreign to normal microspores. No evidence has yet been obtained on the chemical behavior of these substances within the microspores, but if current studies in other tissues provide any pointer, it is that, among other components, they interact with nucleic acids.[6, 7] Recent studies strongly suggest that the principal target of these compounds is DNA. Distortion of DNA synthesis[8, 9] and a shift in melting point[10] are the effects reported for mitomycin C. It is difficult to overlook the fact that inhibitory reagents which act on the synthesis of RNA or protein are ineffective in disturbing regulation. That enzyme synthesis and regulation of that same synthesis are separable processes is patent from these results. What has deeper significance, even though tenuously based, is the possibility that the normal process of regulation, so incisively described by Jacob and Monod[11] can be interrupted by effecting an abnormal complex with DNA. It is important to emphasize the restricted sense in which the phrase "disturbance of regulation" is used. The frame of reference throughout these studies has been the events occurring during an interval of 4–5 days which represents a small slice of cellular interphase. In other studies related to mitosis proper, we have found that microspores treated with azaguanine eventually overcome the inhibition and develop normally after an extended period of delay.[12] Such delay could be justifiably defined as a disturbance of regulation even though the specialized regulatory mechanism is not involved.

Discussion.—As indicated in the introduction to the preceding paper,[1] the aim of these studies has been to throw some light on the general features of mitotic regulation and not on the specific role of thymidine kinase. The aim would be presumptuous were it not for the fact that in other studies of meiosis and mitosis in liliaceous plants the evidence, though still superficial, points in the one direction—that many of the sequential events in this cycle are suppressible by interference with RNA or protein synthesis.[12] Such evidence, together with that from microbial studies on the lack of an obligatory relationship between the presence of kinases or polymerase and *in vivo* replication of the chromosome, make it highly probable that, however integrated a process mitosis may appear to be under normal conditions, it is a composite of individually regulated molecular systems held together by a set of regulatory devices. It is well beyond the scope of this communication to consider whether in any particular type of cell the ultimate regulation of mitosis requires a simultaneous regulation of all the component systems; on *a priori* grounds this need not be so, and on experimental grounds it is probably not so. The tissue chosen for study is perhaps an extreme example of regulation along the time axis of a cell's life, but it is precisely for this reason that the choice was made. The ques-

tion was how intracellular events might be regulated and not which events are so governed. The regulation is given and we have sought to understand it. Whatever understanding has been achieved would have been impossible without the fertile field of reference supplied by molecular studies of biology. The interpretation here provided can at best be no better than the implicated molecular concepts.

In the context of modern molecular studies bridging the potentialities of the genotype with the actualities of the phenotype, one of the challenges presented by the cells of multicellular organisms is to explain how common molecular mechanisms account for their distinctive patterns of behavior. The handmaiden of bacterial growth—the logarithmic plot—gives many metazoan cells only transitory attention. Microspores neatly illustrate the point and make plain the widespread fact that cells have an inherent capacity to alter their physiological complexion along the axis of time. The alteration may lead to division or differentiation; these studies happen to be concerned with division. Underlying the complicated heap of intra- and extracellular factors which govern the life histories of cells, a few basic mechanisms have been made evident in these experiments:

(1) Alterations at a fixed point in time can arise as a consequence of specific gene activation. The conclusion is based on the observations that the characteristics of enzyme appearance are identical with those of induced enzyme synthesis in microorganisms.

(2) Activation of such genes need not be (and, in these studies, is not) a direct response of those same genes to the intracellular environment. This conclusion is based on the dissociability of synthetic activity from the temporal regulation of that activity.

(3) The experiments furthermore point to the probability that interference with regulation is achieved by a complexing or alteration of an already formed nucleic acid molecule. In light of Jacob and Monod's hypothesis, it is tempting to assign the locus of action to the chromosome. On the whole, it would appear that mitosis should be moved from the category of a special mechanism to that of a special expression of a general mechanism.

Summary.—The removal of thymidine kinase some 6–12 hr after its synthesis in lily microspores may be inhibited by subjecting the cells to anaerobic conditions. Regulation of the enzyme-forming and removal system is drastically upset by the following agents: X rays, acridine orange, mitomycin C.

† Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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