*MOLECULAR FACETS OF MITOTIC REGULATION, I. SYNTHESIS OF THYMIDINE KINASE**

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Communicated by David R. Goddard, March 13, 1963

This report, although concerned with a specific enzyme, is aimed at revealing a pattern of behavior which may generally underlie the component events of the mitotic cycle. The experiments have been performed on a specialized group of cells which are antecedents of pollen—the microspores of *Lilium longiflorum* (var. Croft). During the life of the microspore, which extends for several weeks, thymidine kinase activity (and other biochemical activities) appears at a precisely defined time and endures for no more than 24 hours.[1, 2] The periodicity is demonstrable in both intact cells and their extracts. The question raised as a result of this observation concerned the mechanism by which a cell whose interphase spanned several weeks could regulate the appearance and disappearance of a specific enzyme activity in the course of 24 hr. The results which follow provide a partial answer to this question.

Methods.—Lily anthers were cultured by the technique recently described for those of a related genus, *Trillium.*[3] Each group of six anthers was placed in a tube containing 0.2 ml of "Hoagland's medium." The medium is adequate for anthers only at the stages of development studied; earlier stages, or longer growth periods, require a far more complex medium. All cultures were maintained at 15°C by keeping the tubes in a waterbath.

Thymidine kinase was assayed as previously described.[2] Fractionation of microspore proteins for electrophoresis was carried out in the following way: the microspores were homogenized in 0.33 *M* sucrose-0.004 *M* $MgCl_2$—0.05 *M* Tris buffer, pH 7.0 ("SMT" solution) with a Teflon pestle. After checking microscopically for the absence of the whole cells, the suspension was centrifuged at 1,500 $\times$ *g* for 8 min. The pellet was washed once with SMT and the combined supernatant solutions centrifuged at 32,000 $\times$ *g* for 20 min. Analyses of the fractions thus sedimented are not considered here. The supernatant solution was spun at 36,000 rpm for three hr in a Spinco preparative centrifuge using an SW-39 rotor. The soluble fraction was 90% saturated with ammonium sulfate at 0°C and allowed to stand for 20 min, and the precipitated proteins were collected by centrifugation. The clarified solution was lyophilized and checked for enzyme activity. The precipitate was washed once with saturated ammonium sulfate solution, dissolved in 0.05 *M* phosphate buffer (pH 7.6), and dialyzed against *M*/200 of the same buffer 4–5 hr with continuous stirring and 4 changes of outer solution. The dialyzed solution of protein was lyophilized and dissolved in $^1/_{10}$ the original volume of water. It was used as such for electrophoretic resolution. Protein was determined with either the Nessler or Folin reagent.

Results.—Predictability of periodicity in cultured anthers: The sequence and timing of those events studied in cultured anthers are shown in Figure 1. Two features of this sequence deserve emphasis. First, the pattern of periodicity is much the same as that which has been observed on the intact plant; regulation is therefore

independent of the parent plant. Second, the sequence and timing are reproducible. The experiments conducted would have been impossible without the predictability of thymidine kinase appearance within 1 day. For any group of six anthers removed from the plant, the expected time of appearance was computed from the bud length and the regression line (Fig. 1). Since each analysis required the removal of a single anther, intervals between the six possible analyses varied from 12 to 24 hours. Consistent removal of one set of microspores at the point of maximum kinase activity was therefore unlikely. It will be seen that the absolute magnitude of individual peaks varies, although only one peak is present in each group of anthers.

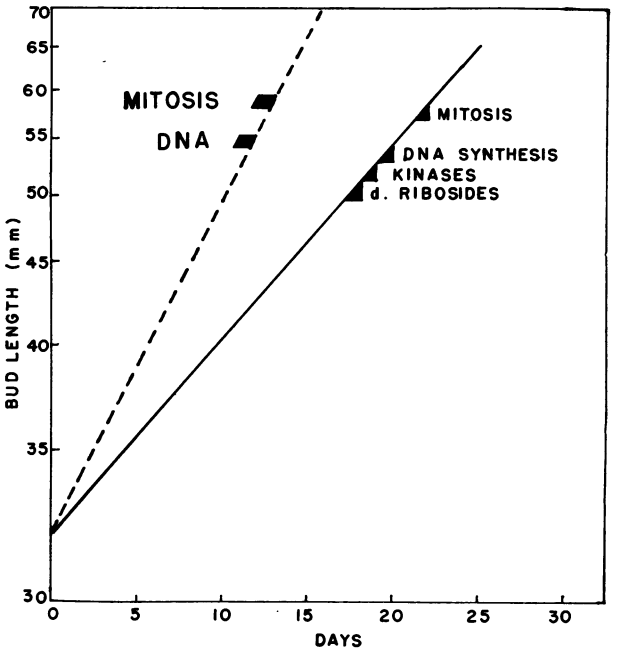

FIG. 1.—Timing of mitotic events in lily anthers cultured at 15°C. The dotted line included for comparison represents the relationships between flower bud length, time of growth, DNA synthesis, and mitosis in the intact plant; it is based on the data of Taylor and McMaster.[7] The times of events indicated on the solid line were experimentally determined: d. ribosides (production of deoxyribosidic compounds); kinases (the appearance of thymidine and thymidylic kinase), DNA synthesis, and mitosis. The usefulness of the slope was made evident by the fact that the occurrence of each of the events could be predicted within ±1 day for anthers removed from buds ranging from 35–48 mm in length.

Penetration of reagents: Unlike the behavior of other plant tissues, anthers show a marked resistance to diffusion of externally added substances into the microspores. This resistance is partly overcome by the technique of culture; pertinent data are given elsewhere.[3] Using isotopic compounds as markers, it has been found that relatively little accumulation occurs during the first 60 hr. Within five days, however, much of the label is removed from the medium, and at such time the fraction of the total label present in the microspores equals or exceeds the ratio of microsporal tissue to that of the anther as a whole. The accelerated accumulation of added reagents following two days of exposure to the medium is characteristic of the amino acids and nitrogenous bases tested. There is, moreover, no close parallel between the extent to which these substances are utilized in syntheses and their rates of accumulation. For the purposes of comparison, three days were arbitrarily set as the time required for added reagents to reach the microspores in effective concentrations. The arbitrariness is unavoidable but is not a serious bar to the interpretation of results.

Effects of inhibitors of RNA or protein synthesis: The first objective was to determine whether the short-lived appearance of enzyme activity during microspore

interphase represented a *de novo* synthesis of enzyme. Paucity of material precluded its isolation and the direct measurement of absolute amounts. Instead, an indirect approach was followed, based upon established evidence of the inhibitory effects of various reagents on protein synthesis. 8-Azaguanine (and later, 5 fluorouracil) were used to distort the synthesis of RNA.[4, 5] DNA would be unaffected since it is not synthesized during the interval stated. Chloramphenicol was used to interrupt the presumed translation of RNA information.[6] 5-Methyl tryptophane and ethionine were introduced to block or distort the pattern of amino acid incorporation into protein. The results of these tests were single and uniform (Fig. 2*A*): all the reagents, if added prior to the normal appearance of enzyme activity, virtually abolished such activity. As might be expected, none of the succeeding steps in the mitotic cycle followed. 32 P-phosphate was not incorporated into DNA even 5 days later than the normally occurring interval of synthesis, nor were any nuclear divisions observed, although the cells were viable. In this set of experiments all reagents were added 8–10 days before the expected appearance of the enzyme. Allowing 3 days for accumulation in the microspores, it may be estimated that the inhibitors were present for at least 5 days prior to the time at which enzyme activity normally appears. If such appearance is due to a synthesis of protein by the commonly accepted sequence of molecular events, then an interval of greater or lesser duration should exist immediately prior to, or coincident with, the appearance of enzyme activity when the reagents are no longer effective. It is possible to specify intervals within an error of ± 1 day, and this is sufficient for our present purpose. It may be seen from Figure 2*B* that if azaguanine is added 4½

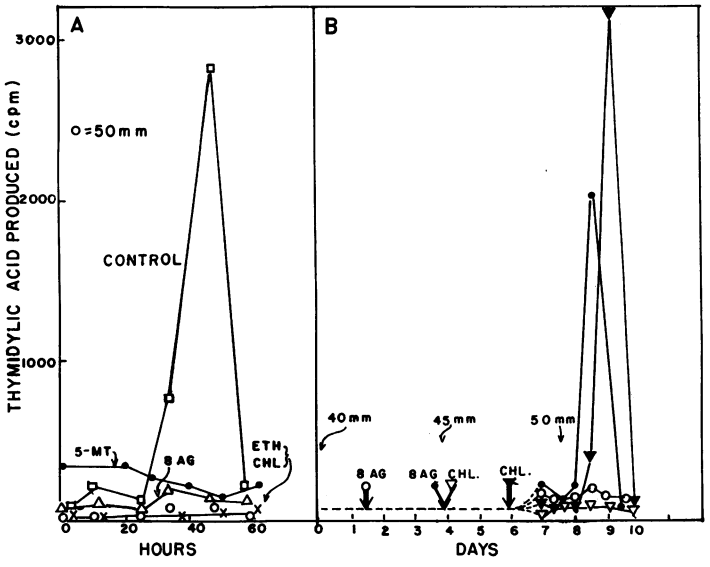

FIG. 2.—Inhibition of appearance of thymidine kinase by various reagents. Anthers were cultured in 0.2 ml of standard medium except for the addition of indicated reagents: 5-methyl tryptophane (5-MT) 0.4 mg; ethionine (eth.), 0.4 mg; chloramphenicol (chl.), 0.04 mg; 8-azaguanine (8-ag), 0.02 mg. Assay conditions described in text. (*A*) Inhibitors added at beginning of culture period. Anthers taken from buds ranging from 36–42 mm in length. The curves drawn are random selections and are typical for the eight or more series run with each inhibitor; in no case did treated microspores show activities above 370 cpm; in most cases the highest value for any set was considerably lower. (*B*) Inhibitors added at times indicated. To interpret these results allowance must be made for the lag in uptake of reagents by microspores which is of the order of 2–3 days. The ineffectiveness of azaguanine and chloramphenicol when added beyond a certain bud length is patent. Ethionine and 5-methyl tryptophane were not tested in this way. Activities are expressed as total counts per microspore from ½ anther.

days before the appearance of the enzyme, it is no longer an effective inhibitor. It would therefore appear that azaguanine must be present in the microspores at least 1–2 days prior to the normal spurt in enzyme activity. Similar remarks apply to chloramphenicol except for the indication that the latter is still effective at a time when azaguanine no longer inhibits. This relationship is so gratifyingly compatible with current interpretations of the molecular sequence in protein synthesis, but so close to the margin of error in our experimental timing, that it is best to consider the result as a fortuitous association rather than as an established demonstration.

The fact that azaguanine had much the same effect, whether added 10 or 4 days prior to the expected appearance of enzyme, made it probable that the process inhibited was of limited duration and occurred no earlier than 2–3 days before the enzyme appeared. If so, then addition of guanine before that interval should reverse the effect of azaguanine irrespective of the time at which the latter was administered. It was first established that guanine itself had no effect on the periodicity of the enzyme and that azaguanine was ineffective if added simultaneously with guanine. The results of adding either guanine or a mixture of guanine-azaguanine at various times after administration of azaguanine alone are shown in Figure 3. The observed behavior thus bears out the interpretation. A secondary effect of the reversal may, however, be noted. Frequently, though not always, the peak of enzyme activity following reversal is broader than the normal. It appears as though the combination has not only restored the ability of the microspores to form enzyme but has somewhat interfered with its regulated removal. This point will be discussed elsewhere.

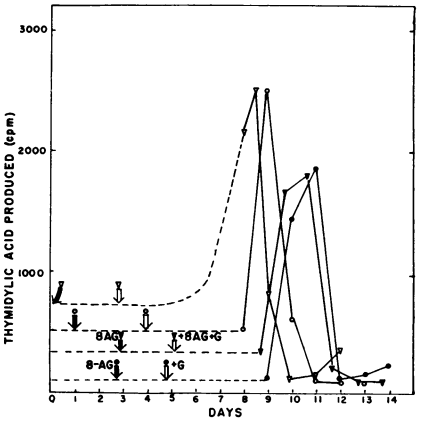

FIG. 3.—Reversal by guanine of inhibitory action of 8-azaguanine. Solid arrows indicate time at which 0.02 mg of azaguanine (8-ag) was added. Circles represent experiments in which anthers were transferred (open arrows) to standard medium saturated with guanine (g); triangles represent those in which anthers were transferred to a mixture of azaguanine and guanine. Guanine alone had no effect. First measurements of activity were made at the beginning of the solid lines. A broadening of the peak may be noted in some of the plots; the effect was frequent.

Incorporation of labeled amino acids in relation to enzyme appearance: Taken together, the first sets of experiments are most simply interpreted as demonstrating that the appearance of thymidine kinase in the microspores is dependent upon a synthesis of RNA and protein. If labeled amino acids are added to the culture medium 8–10 days before the expected appearance of enzyme, and the soluble proteins of the microspores analyzed a few days before and during the interval of enzyme appearance, the ratio of their respective activities is 840:4,250. Differences related to other protein fractions are also evident but they are incidental to the present problem. Chloramphenicol or azaguanine which inhibit the appearance of thymidine kinase also suppress the labeling of the soluble proteins. Results from experiments with labeled amino acids are thus consistent with the conclusion drawn from the first set of studies.

The general correspondence between protein synthesis and thymidine kinase

activity falls short as a demonstration that the enzyme, or part of it, is one of the protein components synthesized. To clarify this point the soluble proteins were fractionated electrophoretically. A large number of anther groups was cultured in the presence of labeled amino acid, and microspores were isolated before, during, and after the expected time of enzyme appearance. The results of this experiment are shown in Figure 4. The most pertinent feature of the graphs is that only during the interval of enzyme appearance do the soluble proteins of the microspores show a labeled component coincident with the position of enzyme activity. For practical reasons it was impossible to determine the specific activity of the protein in the enzyme region. The cellulose acetate strips, when stained with nigrosin, showed a characteristic pattern of bands which, on the whole, changed little during the intervals tested. The positions of the bands provided a convenient way of identifying the location of enzyme, but stain at the locus of the enzyme was undetectable. It may be surmised that protein at the region of enzyme had a comparatively high specific activity, but no quantitative measure of such activity is available. The difference in over-all levels of radioactivity between the curves in Figure 4*A* and 4*B* reflects the fact (noted above) that there is a greater incorporation of label in soluble proteins during the interval of enzyme appearance. The difference in patterns between Figure 4*B* and 4*C* indicates a pronounced synthesis of some other type (or types) of protein following the appearance of enzyme. Since the amount of enzyme activity in *A* or *C* was much too small for detection, the distances from origin and band pattern were used to approximate the position of the enzyme. It is to be

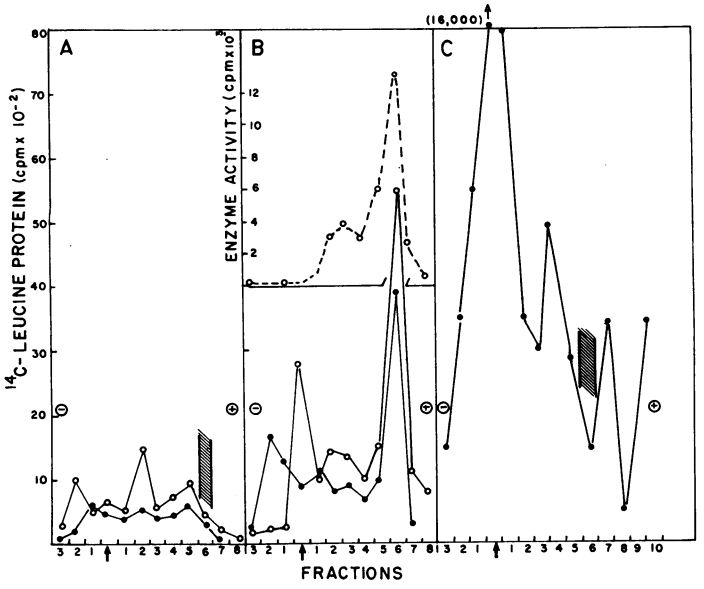

FIG. 4.—Electrophoretic separation of soluble proteins from microspores before (*A*), during (*B*), and after (*C*) appearance of thymidine kinase activity. In order to obtain sufficient material for analysis, microspores from 36 or 48 anthers were pooled for each run. Culture conditions were standard except for the addition of 1 microcurie of ^{14}C-leucine (S.A. = 4 μc/mg) per tube containing 0.15 ml of Hoagland's solution. Buds of approximately 39–42 mm were used in these experiments and the labeled amino acid added at the start. Selection of microspores at desired intervals from each set of anthers was made on the basis of the curve in Fig. 1. Preparation of soluble fraction is described under *Methods*. Negligible amounts of enzyme activity were present in (*A*) and (*C*); shaded bars indicate position of enzyme when present. Cellulose acetate strips (2 cm width) were used in these tests. The position of origin is indicated by a solid arrow. Runs were at 2°C for 6 hr with 1.4 mamps of current and at a pH of 7.4 (0.05 *M* phosphate). Values recorded are total for each pool of microspores. In (*A*) and (*B*) two separate experiments were run, one with 36 anthers, the other with 48.

noted that microspores isolated for Figure 4*C* were exposed to label during the formation of enzyme activity. The experiment was intended to determine whether disappearance of enzyme was paralleled by a disappearance of label in the enzyme region. In this respect the results can hardly be considered decisive. The open circle curve in Figure 4*B* is comparable with that in 4*C* since they represent extracts from the same number of microspores. Thus, to the extent that the level of activity is lower in the region of the enzyme and that the pattern of label distribution has shifted, the results bear out the conclusion that the original enzyme protein has been removed.

In a different set of experiments using 14 C-alanine it was noted that incorporation of label into soluble proteins over an 8-day period was only partly suppressed if either azaguanine or chloramphenicol were added at the same time as the amino acid. The effect was far more pronounced with chloramphenicol, but, since the two reagents appeared to be equally effective in inhibiting the appearance of thymidine kinase, it seemed reasonable to expect in the case of treated cells that virtually no radioactivity would be present on the electrophoretic strip in the region of enzyme if that region contained principally enzyme protein. The curves in Figure 5 make it plain that suppression of enzyme appearance is matched by an absence of label in the enzyme region. Taken together, all the experiments thus far reported point in one direction: a synthesis of either a part or the whole of the enzyme, thymidine kinase. Thus, the likeliest explanation of the initial phase of the phenomenon under examination—the regulated appearance of enzyme activity at a fixed point of interphase—is that the process being observed is an induction of enzyme synthesis.

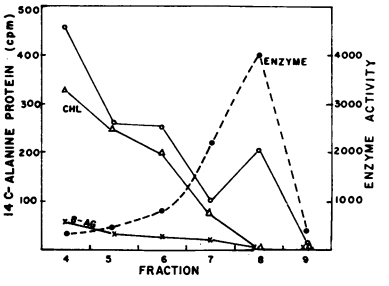

FIG. 5.—Effect of chloramphenicol and azaguanine on incorporation of ^{14}C-alanine into soluble proteins at times when appearance of thymidine kinase is inhibited. Conditions of culture and electrophores are similar to that under Fig. 4, 1 μcurie of ^{14}C-alanine replacing that of ^{14}C-leucine. Microspores of 48 anthers were pooled for each of these experiments. The total soluble protein in each pool was 350 micrograms. In all cases microspores were removed for analysis at the time when enzyme appearance was expected. $^1/_{10}$ of each fraction eluted from acetate strip used for enzyme assay, and values recorded are total counts of thymidylic acid produced.

Discussion.—The target of these studies has been to disclose some of the mechanisms which a cell may utilize to regulate its cyclical behavior. Such studies have been possible because the microspores in lily are synchronized in development and because the sequential events in their life cycle are sufficiently separated in time to permit individual study. It is now apparent that a shift in metabolic processes at a characteristic phase of the cycle has all the earmarks of induced enzyme synthesis. We do not know the nature of the inducer nor its locus of origin. The parent plant plays no immediate role since the microspores undergo a normal cycle of development in anthers which have been put in culture weeks before the occurrence of mitosis. Since no attempt was made to culture the microspores in isolation, it is idle to speculate on whether the stimulus to induction is of exogenous or endogenous origin. What appears to be the most significant aspect of these studies is the evidence that a cell may use identical mechanisms for regulating internal cycles of

development and for adapting to novel nutritional conditions. It seems reasonable to suppose that the periodicity in other biochemical activities of the microspores may be regulated by mechanisms similar to that governing thymidine kinase. If so, then, in the ultimate sense at least, the problem of mitosis is a problem of regulated gene action.

Summary.—The brief appearance of thymidine kinase activity prior to DNA synthesis in microspores of *Lilium* is due to a *de novo* synthesis of protein. The chain of events leading to such synthesis begins with the formation of RNA; any of the steps in the sequence may be blocked by an appropriate inhibitor.

* This work was supported by grants from the National Science Foundation (G15947) and U.S. Public Health Service (GM07897).

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[3] *Ibid.*, *J. Cell Biol.*, **16**, 259 (1963).
[4] Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.
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[6] Aronson, A. J., and S. Spiegelman, *Biochim. Biophys. Acta*, **53**, 84 (1961).
[7] Taylor, J. H., and R. D. McMaster, *Chromosoma*, **64**, 189 (1957).

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*POLYRIBOSOMES IN NORMAL AND POLIOVIRUS-INFECTED HELA CELLS AND THEIR RELATIONSHIP TO MESSENGER-RNA**

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Communicated by S. E. Luria, March 26, 1963

An extensive body of evidence has accumulated in the past two years in support of the hypothesis that protein synthesis is directed by strands of "messenger-RNA" (m-RNA) which are attached to ribosomal particles.[1, 2] From the analysis of attachment to ribosomes of nucleic acids and synthetic polyribonucleotides which stimulate protein synthesis *in vitro*,[3–5] as well as a study of the ribosomal components active in the synthesis of protein in *E. coli*,[6] it appeared that aggregates containing more than one ribosome existed. Examination of the ribosomal complement of rabbit reticulocytes led to the clear demonstration that a unit composed of several (probably 5) ribosomes was responsible for the majority of the protein synthesis.[7–9] This unit was resistant to treatment with sodium desoxycholate, a salt commonly used to dissolve membrane material from animal cell sources, but was readily subject to reduction in size by pancreatic ribonuclease. The conclusion was drawn that the polyribosome or polysome, as the large structure has been called, probably consisted of a strand of messenger-RNA (m-RNA) to which a number of ribosomes were simultaneously attached. A similar conclusion has been reached independently by Wettstein *et al.*,[10] who have studied functioning ribosomal aggregates from rat liver.

An examination of the RNA responsible for protein synthesis in cells derived from whole animals is complicated by the difficulty in using radioisotopes in satis-