TRANSIENT PHOSPHORYLATION OF DEOXYRIBOSIDES AND REGULATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS

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ABSTRACT

Microspores isolated from *Lilium longiflorum* and *Trillium erectum* were studied with respect to their capacities for phosphorylating deoxyribosides *in vitro*. It was found that such capacities are manifest only during brief intervals of time adjacent to periods of DNA synthesis, and that none of the neighboring cells in the anther acquire them. The observed patterns of behavior are interpreted in terms of enzyme induction as a device for regulating DNA synthesis.

INTRODUCTION

In microspores of *Lilium longiflorum* the chain of metabolic events leading to chromosome replication is first marked by a pulse-like production of deoxyribosides outside the cells (1). The abrupt formation of these precursors appears to be due to the induction of deoxyribonuclease activity which persists for an interval of approximately 6 to 8 hours in the region of the anther adjacent to the microspores (2). The results to be reported point to the transient appearance of enzymes associated with DNA synthesis as a general characteristic of metabolic regulation during the mitotic cycle. Specifically, the experiments conducted were concerned with phosphorylation of the extracellularly produced deoxyribosides by an enzyme system within the microspores. On the basis of *in vitro* assays, the enzyme system appeared to be induced close to the time of DNA synthesis. In lily, the enzyme persists for about 10 hours; in trillium, which undergoes mitosis at 2–4°C, it persists for about 2 days.

METHODS

The principal substrate used for measuring phosphorylating activity was C^{14}-thymidine (TDR). It was prepared by incubating a commercial sample of C^{14}-thymine and unlabeled thymidine with extracts of either *Escherichia coli* (strain W^{6-}, kindly supplied by Dr. Seymour Cohen (3)) or *Lactobacillus helveticus* (4). The products of the reaction were separated on Whatman no. 3MM filter paper by partition chromatography using ethylacetate saturated with phosphate buffer as solvent (3). C^{14}-deoxycytidine (CDE) was similarly prepared but was infrequently used because of the strong deaminase activity in the anther (5).

Incubation systems contained the following components (in micromoles): (*a*) Lily: C^{14}-TDR, 0.54×10^{-3} (8000 CPM); adenosine triphosphate (ATP), 2; $MgCl_2$, 4; Sodium-potassium phosphate buffer (pH 7.2), 20; total volume, 0.1 ml. (*b*) Trillium: C^{14}-TDR, 1.08×10^{-3} (16,000 CPM); ATP, 0.5; $MgCl_2$, 0.4; phosphate buffer (pH 7.1), 5; total volume, 0.025 ml. The suitability of the medium used will be discussed under Results. Microspores from one or two anthers were added to the designated mixtures and incubated either for 1.5 hours at 25°C in the case of lily, or 4 hours at 2–4°C in the case of trillium. The reaction was stopped with 2 volumes of methanol and the mixture was centrifuged. The supernatant layer was removed and the residue reextracted with 70 per cent methanol. The combined extracts were concentrated *in vacuo*. The

residue remaining was extracted for 20 minutes with 0.72 M trichloracetic acid at 90°C. Usually all radioactive counts were recovered in the methanolic extracts. Methanolic solutions were spotted on no. 1 Whatman filter paper and the components resolved with a solvent containing *n*-butanol and ammonium formate (5). Radioactivity was measured directly on the paper using a windowless gas-flow counter. Since phosphorylated compounds do not move in the solvent system used, radioactive products remaining at the origin were rerun with an isobutyric acid:ammonia:Versene system (6). The hot acidic extract was placed on a planchet for counting.

Two species of plants were used in these studies. *Lilium longiflorum* (var. Croft) was grown from bulbs in a greenhouse and the anthers were harvested as in previous studies (5). Corms of *Trillium erectum* were obtained commercially and stored in polyethylene bags in a refrigerator at a temperature of 2–4°C. Under these conditions meiosis occurred in mid December and mitosis during the latter part of January or February.

RESULTS

Our first experiments were designed to determine whether enzymes mediating the phosphorylation of deoxyribosides could be found in lily anthers. A variety of developmental stages (measured by bud length) were tested by incubating intact anthers, homogenates of entire anthers or of the wall tissues, and microspores in the presence of C^{14}-TDR. The results were unambiguous on two points: phosphorylating activity was present to an appreciable extent at only a few developmental intervals, and, when present, it was almost entirely restricted to the microspores. Data obtained from a number of experiments are summarized in Table I. No values are given for intact anthers because even when immersed with bases cut they absorbed little C^{14}-TDR during 2 to 3 hours. Absorption and phosphorylation did occur if such anthers were maintained in contact with isotope

TABLE I

Thymidine Phosphorylation in Anthers of Lilium

Tissue	Bud length				
	24 mm	30 mm	37 mm	46 mm	52 mm
Wall (homogenized)	122	80	0	66	0
" (sliced)	0	0	0	0	0
Microspores (unwashed)	250	57	60	370	3745
" (washed)	235	61	55	355	3750
Microspore washings	0	0	0	0	0

Activities are expressed as counts per minute of thymidylic acid per anther after a 2 hour incubation at 25°C. Conditions of experiment described under Methods. Intact wall tissue was prepared as outlined earlier (5) and sliced transversely in sections 1 mm thick. Such slices, like intact anthers, fail to form any thymidylic acid.

TABLE II

Effect of Phosphate on Dephosphorylation of Thymidylic Acid by Lily Microspores

Conditions	Bud length					
	43.5 mm		52.0 mm		59.3 mm	
	1.5 hr	6 hr	1.5 hr	6 hr	1.5 hr	6 hr
0.1 M phosphate	720	2730	600	1870	75	500
0.2 M phosphate	0	0	0	0	0	0
0.2 M phosphate + ATP	0	0	0	0	0	0

Incubation medium as indicated under Methods except for phosphate concentration and the omission of ATP. Approximately 3000 CPM of thymidylic acid were added to unwashed microspore preparations and incubated for the times indicated at 17°C. The products were separated on paper chromatograms. Values are reported as CPM of thymidine produced per anther. Similar results were obtained by using amounts of unlabeled thymidylic acid large enough to be measured spectrophotometrically.

TABLE III

Effect of Sucrose on Thymidine Phosphorylation by Microsporocytes and Microspores of Lily and Trillium

Additions	Lily (52 mm)	Trillium					
		Pachytene	Early interphase (microspores)				
	2 hr	4 hr	1 4 hr	2 4 hr	2 8 hr	3 4 hr	3 8 hr
None	1820	528	1000	530	—	330	—
Sucrose (0.35 M)	130	277	110	130	270	130	270
Sucrose (0.35 M) after preincubation	—	500	—	320	870	330	670

Conditions of incubation as described under Methods. In the third set of tests solid sucrose was added following 1 to 2 hours incubation in standard medium less thymidine. Values are expressed as CPM of thymidylic acid; dashes indicate no measurement.

for 1 or more days, but in view of the timing pattern sought, experiments of this type had little value. Some phosphorylation of thymidine occurred in wall tissues, but at bud lengths greater than 37 mm this is largely due to microspore contamination. At bud lengths below 30 mm phosphorylative activity in the wall tissues, though small, cannot be attributed entirely to contamination by microsporocytes, and is probably associated with the DNA-synthesizing mechanism of the tapetal tissue. Whatever the meaning of these low activities, the significance of the fiftyfold or greater increase near the interval of DNA synthesis is clear enough, the more so as this increase is confined entirely to the microspores.

A number of experiments were conducted to clarify certain biochemical features of the test system used. Initially we were concerned with phosphatases which are present at most stages of anther development and rapidly hydrolyze either added deoxynucleotides or those formed in the course of incubation. Although these enzymes are present mainly in regions outside the microspores, we have been unable to remove them completely from the microspore fraction. Their effect, however, was eliminated by use of 0.2 M phosphate buffers which suppressed phosphomonoesterase, phosphodiesterase, and deoxyribonuclease activities (Table II).

The tonicity of the assay medium is another factor affecting enzyme measurements. Originally it was expected that a medium which maintained the microspores intact would provide optimal conditions for enzyme activity. It was found, however, that media containing concentrations of sucrose (0.35 M) which entirely prevented cell rupture yielded appreciably lower phosphorylating activities than media in which sucrose was omitted. That such behavior might be due to membrane contraction in the hypertonic medium was suggested by the absence of any inhibitory effect when sucrose was added together with labeled substrate after the microspores had been incubated in a sucrose free medium. It would appear, therefore, that some disruption of the microspore is necessary to demonstrate phos-

TABLE IV

Phosphorylation of Thymidine in Presence of ATP by Suspensions of Lily Microspores (53.0 mm Bud Length)

Additions	Unwashed	Sucrose-washed	Washed by incubation medium
None	98	57	155
ATP	1580	1400	1680

Suspensions were incubated for 1.5 hours at 21 °C in the medium described under Methods except for the omission of ATP in the one set of tests. Values are for CPM of thymidylic acid per anther. Similar results were obtained with preparations from trillium. In general, microspores show a marked response to ATP although occasionally a preparation may have sufficient endogenous substrate to mask the effect of ATP.

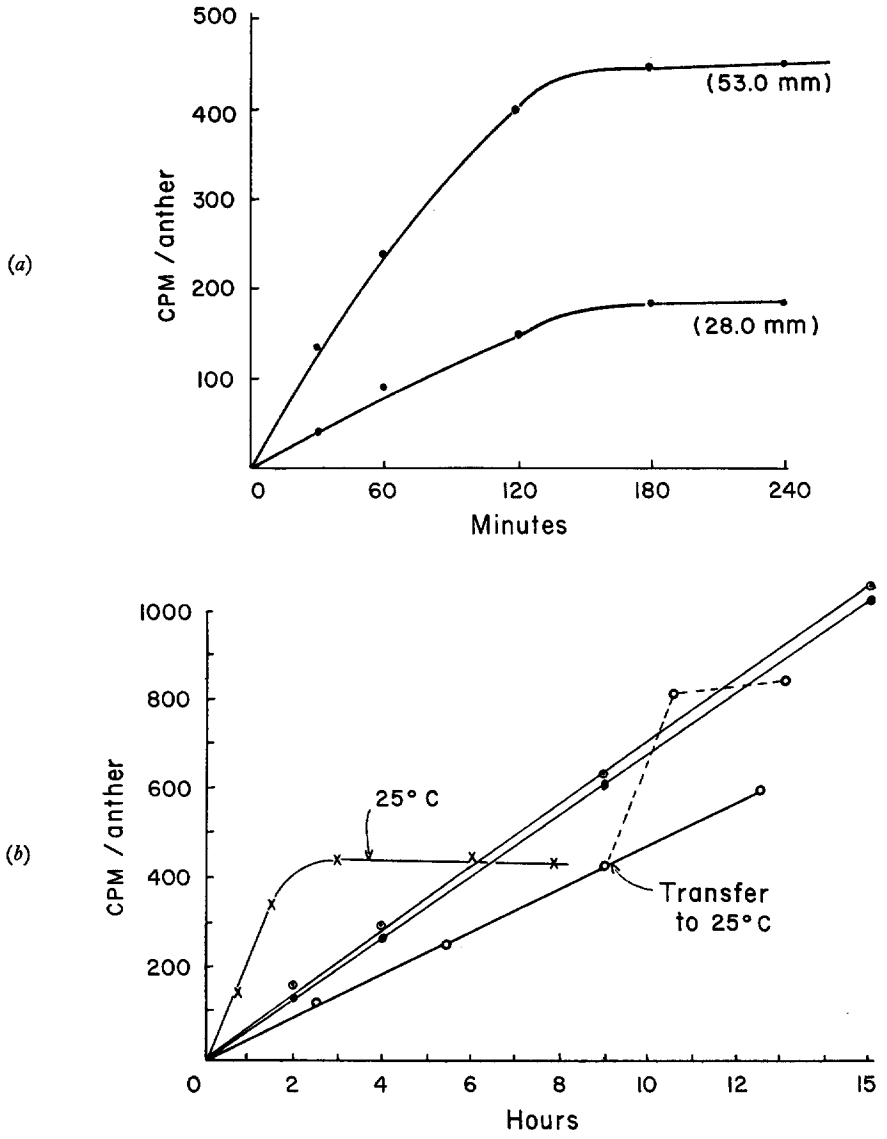

FIGURE 1

Phosphorylation of thymidine by microspores of lily and trillium. Each curve is based on analyses of anthers from the same bud. In the case of the "transfer" experiment in trillium, a portion of the suspension was removed after incubation at 4°C and transferred to 25°C. *a*, lily; *b*, trillium.

phorylations. Whether the cell surface or some internal system is the effective site of disruption remains unknown (Table III).

A third biochemical feature of the test system relates to the role of ATP as a phosphate donor. It was essential to our experiments that non-limiting amounts of such a donor be present at all times; otherwise no distinction could be made between fluctuations due to limitations of substrate and those due to variations in enzymatic activity. To clarify this point a number of experiments were set up in the following way: About 8 lily buds of lengths close to the interval of DNA synthesis were cut under water at 2 inches below the base of the perianth. Buds thus removed were kept with stems submerged in a beaker of water

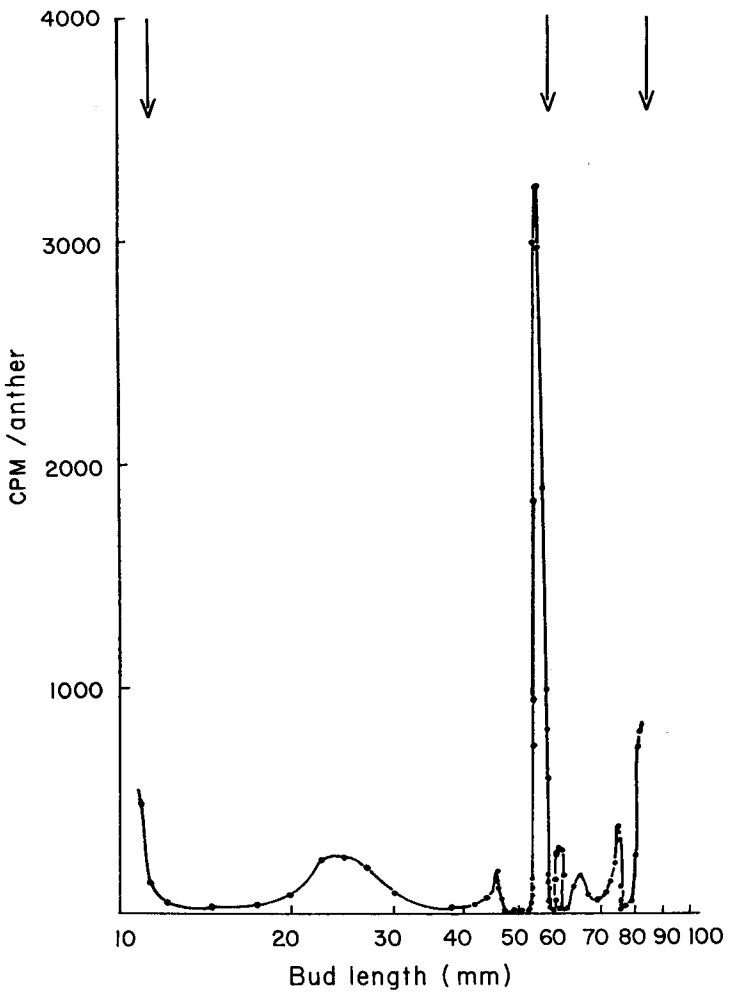

FIGURE 2

Periodicity of thymidine phosphorylation by suspensions of microsporocytes and microspores of lily. Vertical arrows indicate approximately the intervals of DNA synthesis. Tetrads resulting from meiosis appear at 24 to 25 mm bud lengths. Mitosis occurs in the region of 62 to 63 mm.

and after a single anther had been excised from each, they were stored at 4–5°C. Cold storage halted development, so that after microspores had been tested for phosphorylating activity, the "active" buds could be selected (generally there were 1 or 2 of these per group of 8) for the desired experiments.

The result of these experiments (Table IV) was to demonstrate that ATP could serve as a phosphate donor for deoxyriboside phosphorylation. In the particular experiment reported, the microspore fraction contained little endogenous substrate; other microspore preparations, by contrast, had enough endogenous phosphate donors to show little effect of added ATP. In all cases, however, washed microspore fractions showed much the same response to ATP provided they contained active enzyme. Variations in levels of added ATP, alterations of medium tonicity, or addition of washings from "active" microspores failed to elicit any response in these microspores which showed no enzyme activity in the standard test medium.

Rates of phosphorylation with respect to in-

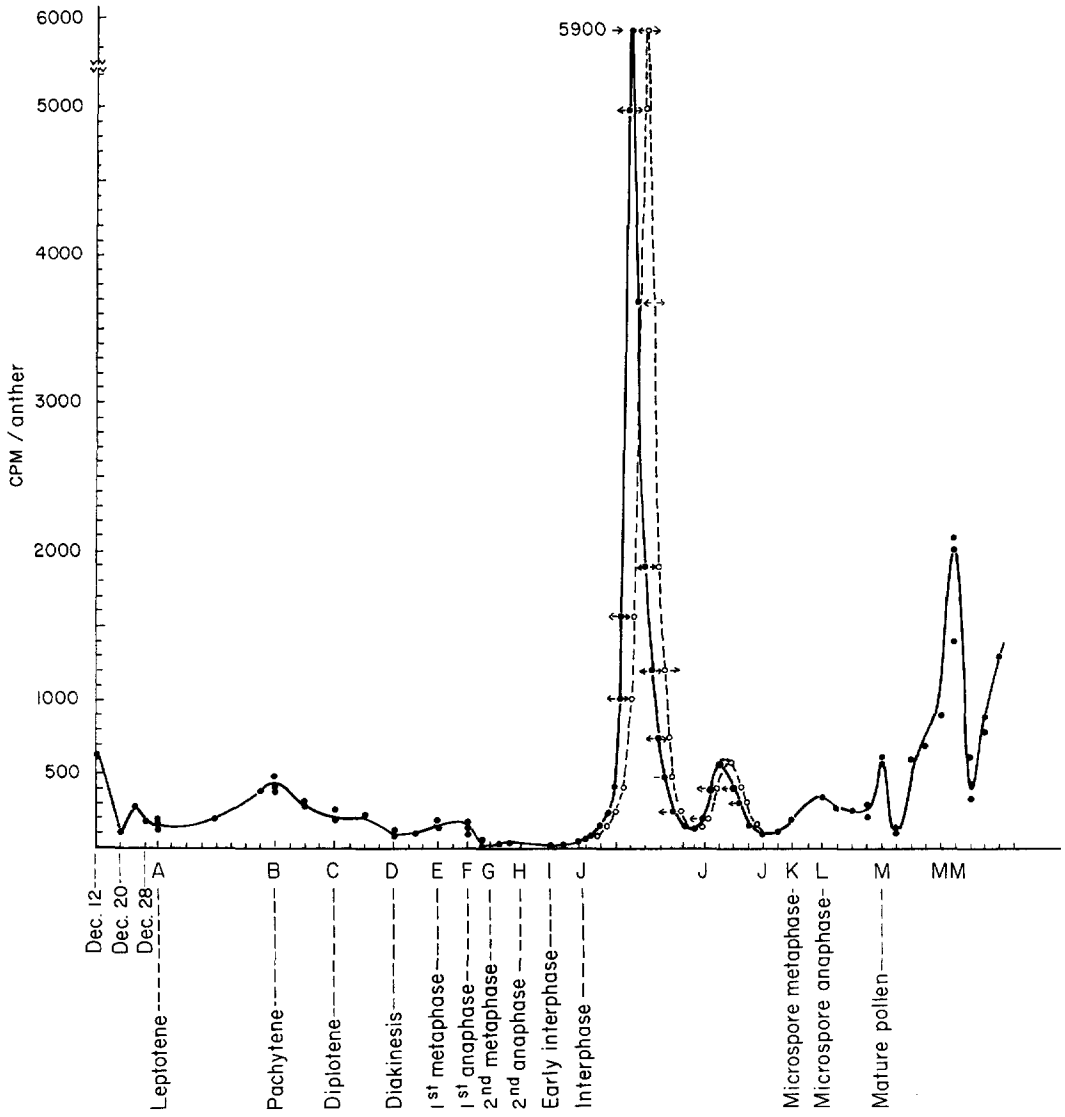

FIGURE 3

Periodicity of thymidine phosphorylation by suspensions of microsporocytes and microspores of trillium. Stage *MM* probably represents interval close to postmitotic DNA synthesis. The *J* interval represents interphase. Time separation within that interval is approximate, and was arrived at (*a*) by recording the date at which each anther was removed from a group of plants kept close together during storage, and (*b*) by removing anthers sequentially at 2 day intervals from the same bud. The small horizontal arrows are meant to denote this uncertainty in timing.

cubation time were also examined. In the case of lilies, it was found that a plateau is reached after 3 hours but that a linear rate prevails during the first 2 hours (Fig. 1). Phosphorylation in lily microspores was therefore assayed for a period of 1.5 hours. In trillium, on the other hand, microsporocyte and microspores maintained a linear rate for intervals as long as 13 hours when incubated at 4–5°C, the temperature of their normal environment.

The periodicity of the enzyme system is illustrated for microspores of lily and trillium in Figs.

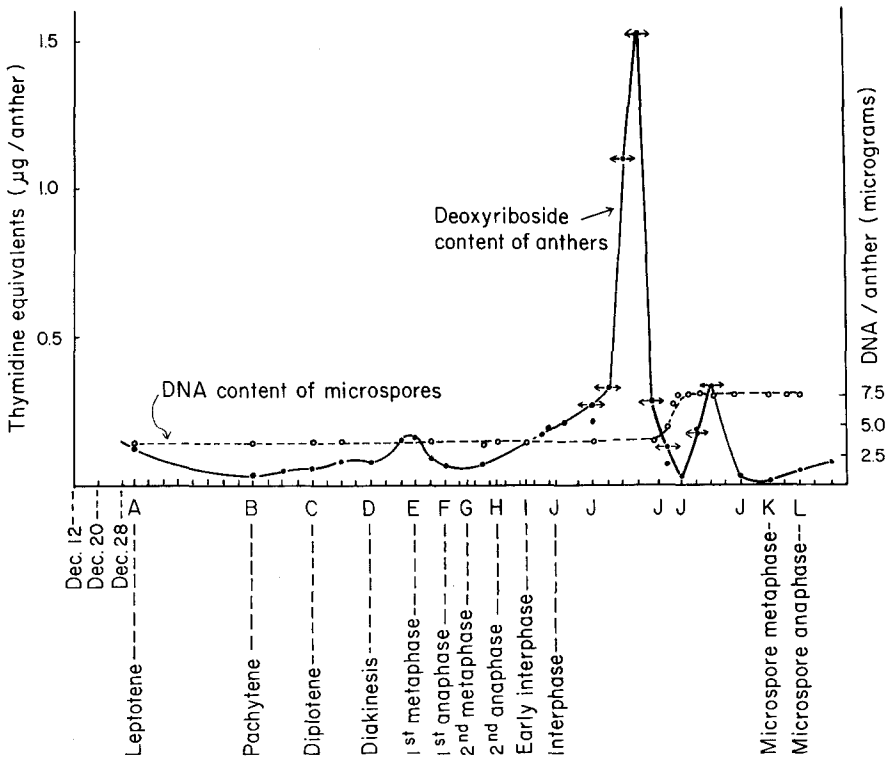

FIGURE 4

Deoxyriboside production in anthers of trillium and DNA content of microspores. Remarks concerning abscission made under Fig. 3 also apply here.

2 and 3. For both plants, the most striking feature of the curves is the steep rise in enzyme activity preceding the DNA synthesis interval associated with mitosis. There are indications of minor peaks apart from those representing incomplete analyses of other synthetic intervals. Such peaks do not lend themselves readily to interpretation except as they suggest that some phosphorylations are necessary during meiosis and mitosis after DNA synthesis is completed. The important point of these results is that under apparently optimal *in vitro* conditions, active phosphorylating enzymes can be found only during limited spans of time in the developmental cycle of the cells. It may be supposed that some inactive form of the enzyme is continuously present; it must be concluded, however, that, irrespective of its nature, the mechanism producing active enzyme is regulated in time. In the case of lily, it is evident from previous plots of deoxyriboside pools that the interval of enzyme activity is close to that of deoxyriboside production (1). The same holds true for trillium, as is shown by plots of deoxyriboside pools and DNA content in Fig. 4.

One point of importance is the duration of enzyme activity. In a previous study, deoxyribonuclease was found to persist for about 8 hours (2). A similar situation prevails with respect to phosphorylation. In Fig. 5 are plotted the phosphorylating activities of microspores obtained from anthers removed sequentially in a manner described earlier (2). In lily, the duration of phosphorylation is of the order of 12 hours; in trillium, at 2–4°C, it is about 2 days. In both cases the striking point is that enzyme activity is found only over a relatively short interval of the mitotic cycle. Thus, quite apart from the temporal association between phosphorylation and DNA synthesis, it would appear that one characteristic mark of a developing cell is its capacity to form and remove a particular enzyme activity.

One other feature of the phosphorylating pat-

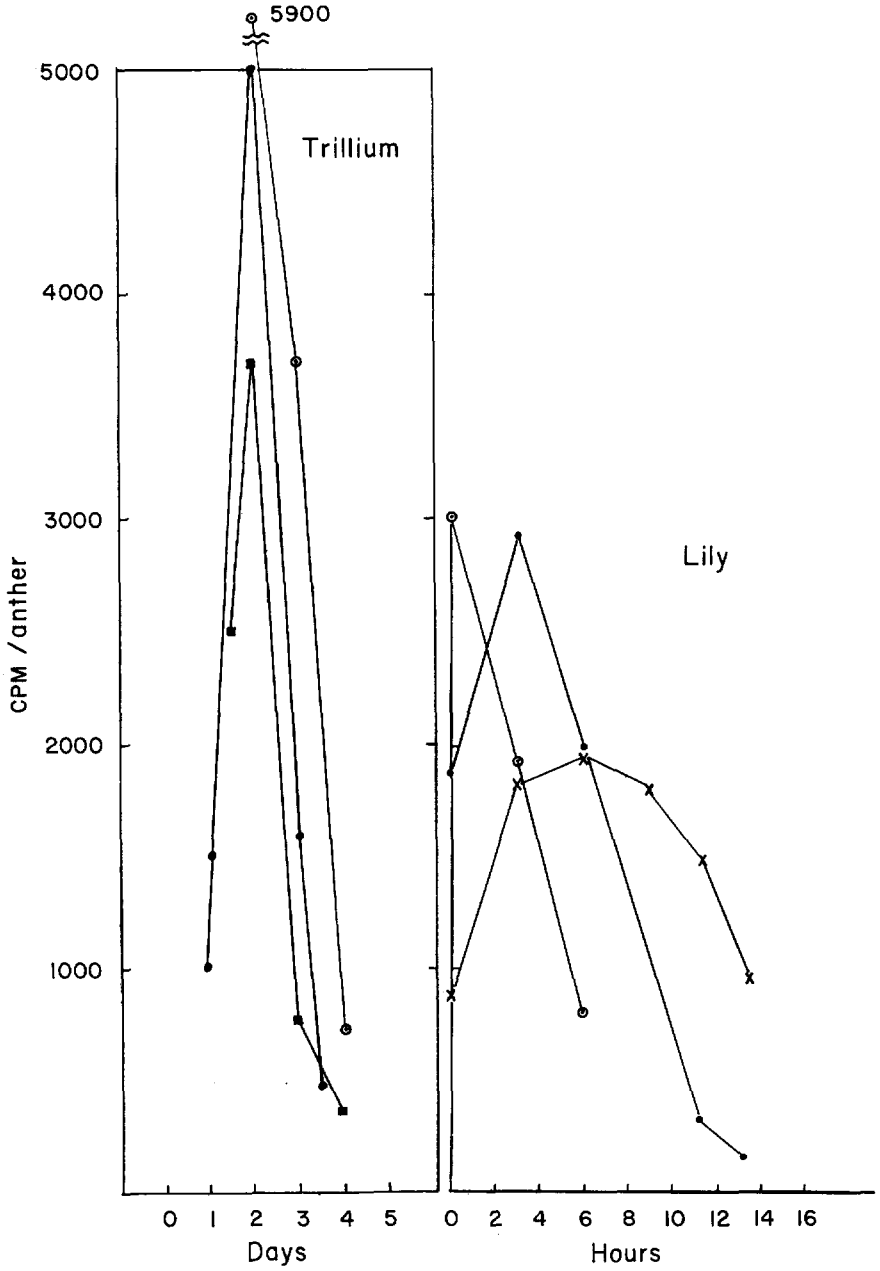

FIGURE 5

The duration of thymidine phosphorylation cycles in microspores of lily and of trillium. Each curve represents the activities of microspores obtained from a single bud. Details of experiment in text.

tern calls for mention. Commonly, the reaction observed was a conversion of thymidine to thymidylic acid. It would be misleading, however, to suppose that the cycles here reported cover that reaction alone. Since reaction products were differentiated on both butanol-formate and isobutyric-ammonia paper chromatograms, the nature of the phosphorylated derivatives could in each case be specified. In some instances, notably those occurring in the declining phase of the cycle, pyrophosphate derivatives were also found. The inference we tentatively draw is that pyrophosphorylation follows but partly overlaps the initial phosphorylating activity. Since we intend to study the latter reaction using phosphorylated deoxyribosides as substrates, the observation will not be pursued here.

DISCUSSION

The evidence we have reported in this and preceding publications (2, 5) may be summarized thus: Those enzymatic activities associated with the production of deoxyribosides and their phosphorylation are present only during brief intervals adjacent to DNA synthesis. Other enzymes, such as deoxycytidine deaminase, phosphodiesterase, and phosphomonoesterase, are present throughout development. Since we have been unable to find evidence for the presence of inhibitors in nonactive cells, we suppose that enzyme activity is periodically induced. We have not determined whether the induction in question represents enzyme synthesis or proenzyme modification, but in so far as these results serve as pointers, they point in the same direction as microbiological studies of adaptive enzyme formation. The direction we have in mind is that of the control of cell metabolism at the level of enzyme-forming systems. If this be true, then the so-called mitotic "trigger" is no more than a special instance of the "inducer" concept occasionally applied to explain differentiation. And, if current thinking about the intimate association between inducer and gene is correct, then we must suppose that whatever the complexity of controlling factors in a cell, initiation of chromosome duplication has its seat in the chromosome itself.

Any attempt to translate the pattern of metabolism observed in microspores into general terms requires, at the least, a large number of qualifications. To begin with, in many bacterial cells, DNA synthesis is not restricted to a portion of interphase (7); in such cells the regulatory mechanism appears to reside in the induction of cytokinesis which follows doubling of the DNA complement. It is quite possible that most cell populations growing logarithmically simply maintain a constant and adequate level of enzyme activity. Furthermore, even in those cells which synthesize DNA periodically, a demonstrable point of regulation is at the site of the chromosome itself, different portions of which duplicate at different intervals (8, 9). Thus, if our interpretation of microspore behavior is correct, it could be meaningfully extended only to those cells in which DNA synthesis is limited by enzymes acting on DNA precursors. One facet of microspore behavior nevertheless has general significance: the capacity of a cell to form and remove enzyme activity during a brief interval of its life cycle.

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