PERIODIC INDUCTION OF DEOXYRIBONUCLEASE ACTIVITY IN RELATION TO THE MITOTIC CYCLE

HERBERT STERN, Ph.D.

From the Research Branch, Canada Department of Agriculture, Ottawa. Dr. Stern's present address is Department of Botany, University of Illinois, Urbana

ABSTRACT

The behavior of developing anthers has been studied with respect to deoxyribonuclease. This enzyme, in contrast to related hydrolytic ones, is sharply periodic in its activity. Whenever a pool of deoxyribosides appears *in situ*, it is preceded by the appearance of deoxyribonuclease. The duration of pool or enzyme does not exceed and is generally less than 12 hours, even though the life span of the cells concerned is of the order of 25 to 30 days. The significance of periodic enzyme induction is discussed in relation to cell morphogenesis.

INTRODUCTION

The requirement of deoxyribosides for deoxyribonucleic acid (DNA) synthesis is patent. In this connection, two problems exist—the mode of deoxyriboside synthesis and the regulation of such synthesis with respect to time. Much information is already available on the first of these questions, and although the picture is incomplete, it will not be considered here. It is to the problem of regulation that this communication is addressed.

The periodic production of deoxyribosidic compounds associated with chromosome reproduction in lily anthers has been described (1). A similar periodicity has been observed in synchronously dividing bacteria (2). The question now posed in whether such pulse-like production of DNA precursors is matched by a corresponding pattern in enzyme activity. To answer the question it has been necessary to select a pertinent enzyme system, bearing in mind that in this connection its specific catalytic property is of secondary importance to its regulatory behavior. Deoxyribonuclease (DNase) was the enzyme chosen as a result of our observation that microsporal DNA synthesis occurred during a period of decline in total DNA content of the anther and that this period was punctuated with intervals of DNase activity (1).

The substance of our results is that pulses of DNase activity persisting from 4 to 8 hours occur in the course of anther development and that such pulses are initiated prior to deoxyriboside formation. We do not know the locus or nature of the DNase substrate, nor can we account for the total decline in anther DNA in terms of the pulses identified.

METHODS

On the whole, methods and materials used were similar to those reported in a preceding publication (3). Phosphatase was measured by incubating tissue homogenates with appropriate deoxyribonucleotides and separating the reaction products on a butanol-water paper chromatogram. Phosphodiesterase was determined by means of the artificial substrates, sodium di-*p*-nitrophenyl phosphate synthesized according to Corby *et al.* (4), or the calcium salt obtained commercially. The method followed was that of Sinsheimer and Koerner (5), modified so as to allow for the fact that anther diesterase has a low pH optimum and does not respond to Mg^{++} (1). Thus a 0.01 M stock solution of dinitrophenyl phosphate was made up in 0.01 *M* acetate buffer (pH 4.7). Before use, a portion of the solution was diluted threefold with water and 0.75 ml. of it pipetted into a tube along with 0.25 ml. of 0.5 M acetate buffer of desired pH and 0.1 ml. of tissue extract. The mixture was incubated at 25°C. and at 0, 10, and 20 minute

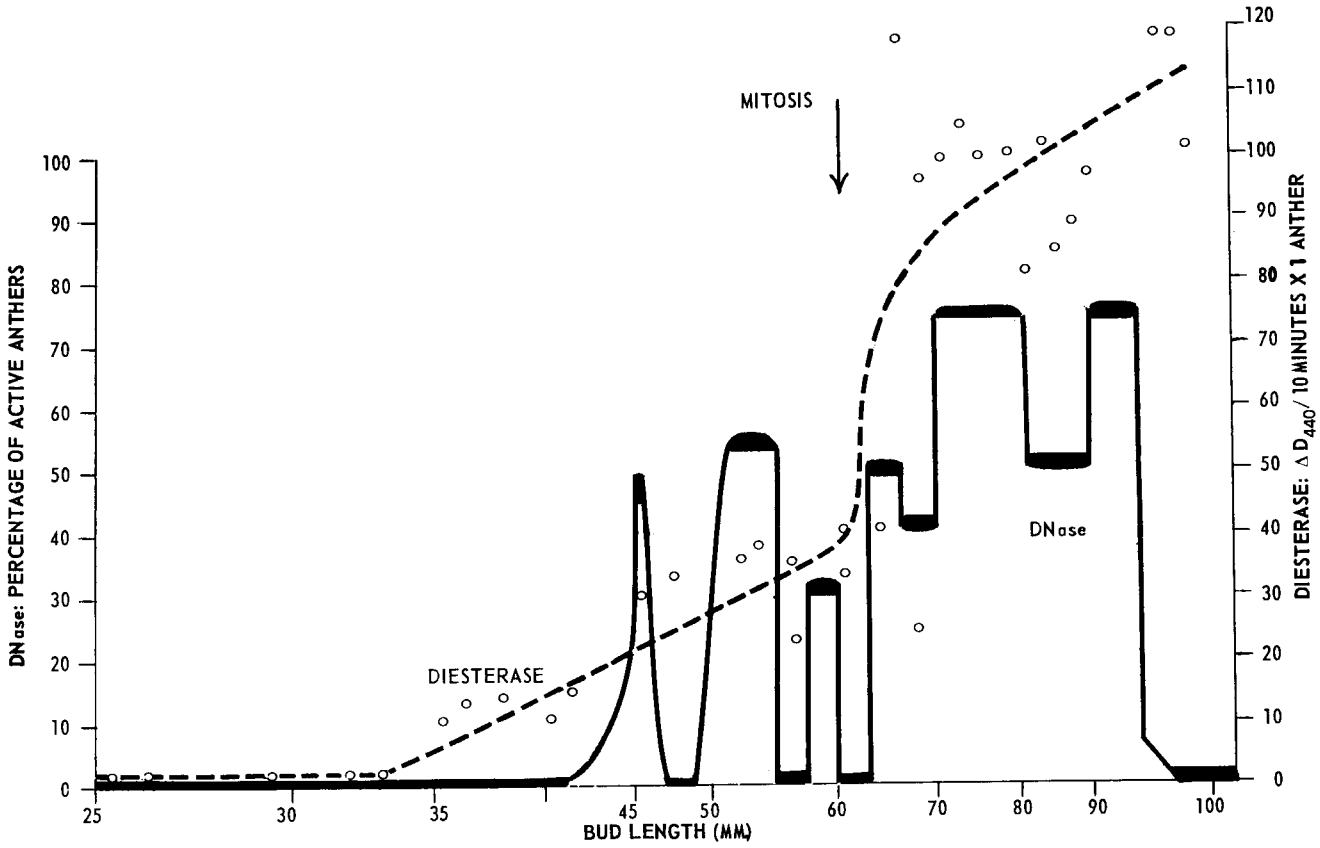

FIGURE 1

Variations in phosphodiesterase and DNase activities during the course of anther development. Conditions described under Methods. Note the difference in ordinates for DNase and diesterase respectively. Open circles are averages for anthers from 1 to 3 buds. Thick lines represent groups of 4 to 12 buds over intervals indicated. Although for comparative purposes similar plots of DNase and diesterase activities would appear to be desirable, the graphic result would be confusing. Dots representing low and high DNase activities would be scattered over the figure and thereby obscure the striking point that even over a 1 mm. interval, some anthers have a low and others a high activity. See Table I.

intervals 0.25 ml. was pipetted into a cuvette containing 100 ml. of 0.2 N NaOH. The absorption was immediately read at 440 mμ. DNase was measured either by following the formation of acid-soluble products from polymerized calf-thymus DNA (6) or by assaying microbiologically the production of deoxyribosides from endogenous substrate (7).

RESULTS

Developing anthers are the seat of DNase, phosphodiesterase, and monoesterase activities, the sum of which would account for the production of deoxyribosides from some form of polydeoxynucleotide (1). The set of measurements which therefore engages our attention is the variation in activities during anther development. Phosphomonoesterase was not studied systematically, but scattered observations indicate its pattern of behavior to be similar to that of diesterase. There is, however, a striking difference in the respective developmental patterns of diesterase and DNase (Fig. 1). The one increases continuously; the other fluctuates widely. Mature anthers are a rich source of diesterase. Dr. Y. Hotta has made some initial purifications of the enzyme and found it to be separable from the monoesterase present. The partially purified enzyme, like the crude extract, is completely inhibited in phosphate buffer.

The curve for DNase is a plot of the percentage of anthers in a given interval of bud length having an activity above an arbitrary established minimum. This arbitrariness does little to bias the picture as the active group breaks down 40 to 80 μg. DNA-P/hour, whereas the inactive one varies within the range of 5 to 15 μg. (Table I). Intermediate values are found but they are infrequent. It will be observed that among the 80 buds thus tested here, those below 43 mm. and above 105 mm. had no appreciable DNase activity in their anthers. The interval spanning these two lengths is marked by large fluctuations. At some points, no DNase activity was observed; at others, a variable percentage of the anthers were active. Although the fluctuations correspond roughly to intervals of deoxyriboside production, such correspondence is clearly insufficient to provide for a definitive relationship. The spread of the fluctuations can be attributed in part to the large changes in greenhouse temperature over the period studied (1), but the only conclusion which can be reasonably drawn is that at certain defined intervals of growth DNase may or may not be present in an anther. Obviously, the question to be answered is why all-or-none fluctuations occur in DNase but not in related hydrolytic enzymes.

TABLE I

DNase and Diesterase Activities in Pre-Mitotic Anthers

Bud length	DNase	Diesterase
45.5	68	39
47.5	6	30
49.6	72	40
49.7	10	40
51.9	56	36
52.6	8	39

DNase activities are expressed as μg. DNA-P/anther × 1 hour. Diesterase activities are expressed as Δ O.D. $\frac{1\text{cm.}}{440\text{m}\mu}$/10 minutes for a solution containing the extract of one anther per ml. Assays were run at 25°C. The limited interval of bud length here chosen is meant to illustrate the difference in behavior of the two enzymes.

Our first effort was to test for the *in situ* presence of deoxyribosides simultaneously with and 18 hours after DNase assay. We supposed that if DNase were tied to deoxyriboside production in the living anther, deoxyribosides should be found either while the enzyme is active or afterwards. The idea was tested by removing one anther from a bud and assaying it for DNase, removing a second for measurement of the internal deoxyriboside pool, and culturing the remainder, which had been excised with filaments intact, in a drop of buffer overnight at 15 or 25°C. Duplicate sets of the cultured anthers were analyzed in the same way as the initial ones. The results provided little enlightenment. Anthers with initially high DNase activity had much the same activity after 18 or even 40 hours in culture, making it appear unlikely that the variable percentages shown in Fig. 1 were due to brief periods of enzyme activity. Moreover, the presence or absence of deoxyribosides appeared unrelated to the presence or absence of DNase either at the time of its measurement or 18 hours later.

There were two shortcomings, however, to the above experiments. First, we could not be sure that the cultured anther would behave like the *in situ* one. Second, we had no reason to presuppose that 18 hours was a meaningful interval. It could also be argued that anthers of a single bud were not necessarily at similar stages of de-

TABLE II

Endogenous Production of Deoxyribosides by Anther Homogenates

Micrograms thymidine eequivalents per anther

Bud length	52.0 mm.			64.3 mm.			67.6 mm.			71.8 mm.		
Time, *hrs.*	0	½	1	0	½	1	0	½	1	0	½	1
Treatment:												
Acetate alone	0.8	0.5	1.7	—	0.6	0.7	—	3.0	6.0	.2	15.0	23.0
Acetate, then sucrose	1.1	0.6	1.5	—	0.5	0.4	—	2.6	5.0	.3	13.0	17.0
Sucrose alone	1.0	0.3	0.8	—	—	—	—	1.5	3.0	.1	13.0	15.0
Sucrose, then acetate	0.5	0.2	1.0	—	0.8	1.7	—	3.6	4.9	.1	12.0	15.0

Conditions for assay described in text. — indicates a value of less than 0.2 μg. per anther.

velopment, but our experience in measuring deoxyriboside pools of individual anthers led us to believe that this could not be a significant factor in the experiments.

To avoid the above shortcomings a flap was made in each bud by two longitudinal slits and the anthers removed for analysis at 2 to 3 hour intervals. During interim periods the buds were enclosed in polyethylene bags and the plants kept in the greenhouse. To facilitate enzymatic measurements, it was decided to use the endogenous DNA of the anther as substrate. A number of tests had to be carried out, however, in order to establish the reliability of the method.

Essentially, the method was to measure the amount of deoxyriboside released by homogenized anthers over a given period of time. The effects of tonicity and pH of the medium were first tested. Anthers of a single bud were slit longitudinally and corresponding halves were pooled in two groups. One group was homogenized in 0.1 M acetate buffer (pH 5.25), the other in 0.35 M sucrose. Enough solid sucrose was added to a portion of the acetate homogenate to make a concentration of 0.35 M, and enough 10 M acetate buffer added to a portion of the sucrose homogenate to make it 0.1 M with respect to acetate. The four suspensions were incubated at 25°C. for different intervals of time and the reaction stopped by addition of trichloroacetic acid to a final concentration of 0.36 M or of absolute methanol to a final concentration of 70 per cent. The supernatant fluids obtained upon centrifugation were assayed microbiologically for deoxyriboside content. Results of the experiments are summarized in Table II. It is apparent that the amounts of deoxyribosides released by anthers of the same bud are similar. It is easy to distinguish between those which release appreciable amounts of deoxyriboside and those which do not by any of the four procedures. In view of this, it seems unlikely that the appearance of DNase is due to osmotic shock of subcellular particles. This is in line with the observation that fluctuations in DNase activity (as measured by diphenylamine analysis) occur even though the anther homogenates were diluted fifteenfold with water for measurement. The 0.35 M sucrose was chosen as the simplest test medium.

A comparison was next made between the release of deoxyribosides in a sucrose suspension and the capacity of suitably modified portions of these suspensions to degrade polymerized calf-thymus DNA. The results are reported in Table III. The coincidence of the two methods is clear. Although far more deoxyribosidic material is released in the presence of added substrate (to compare the two columns, thymidine equivalents should be divided by 7.8), the differences between active and inactive buds are adequately defined by either procedure. It might be added that treating the endogenously derived soluble products with diesterase does not appreciably alter their microbiological activity, a result which would be expected in view of the continuous presence of diesterase in anther extracts.

In our first set of experiments, two anthers were removed initially, then grouped as described. One group was assayed for DNase, the other was homogenized in 70 per cent methanol and assayed

TABLE III

Endogenous Deoxyriboside Production and DNase Activity of Anther Homogenates

Bud length	Deoxyribosides produced	DNase	Bud length	Deoxyribosides produced	DNase
53.6	17	30	73.8	—	—
58.2	—	—	79.5	8	31
59.5	—	—	80.6	26	44
61.2	—	—	81.0	11	25

Deoxyriboside values are for micrograms thymidine equivalents released per anther in one-half hour at 25°C. DNase values are for micrograms DNA-P released from polymerized calf-thymus DNA under similar conditions. — indicates less than 3.1 μg. thymidine equivalents or 5 μg. DNA-P.

for deoxyriboside content. Single anthers were removed at subsequent intervals and only their deoxyriboside content measured. 12 of the 16 buds thus tested showed no DNase initially, and no significant content of deoxyribosides over a period of 9 hours; a few showed an increase 24 hours later. The course of the deoxyriboside pool in the few buds with initially high DNase activity is shown in Fig. 2. Deoxyribosides appear in all cases but they may lag in appearance and do not persist for periods longer than 12 hours. The results made it apparent why measurements of deoxyriboside content simultaneously with or 18 hours after that of DNase do not yield meaningful relationships.

In our second set of experiments we sought to characterize the cycles of both DNase activity and deoxyriboside production. To do this, anthers were removed successively and split longitudinally, one-half anther being used for enzyme assay, the other for deoxyriboside content. Three typical examples have been chosen for illustration in Fig. 3, among those buds in which deoxyribosides were found only *after* the beginning of the experiment. Apart from those which showed neither DNase activity nor deoxyriboside formation throughout the course of the experiment, there were, of course a variety of situations which may be found by picking any point along the time axes in Fig. 3. The rule which has thus far proved to be invariant is that the appearance of deoxyribosides is preceded by DNase activity. A few situations have

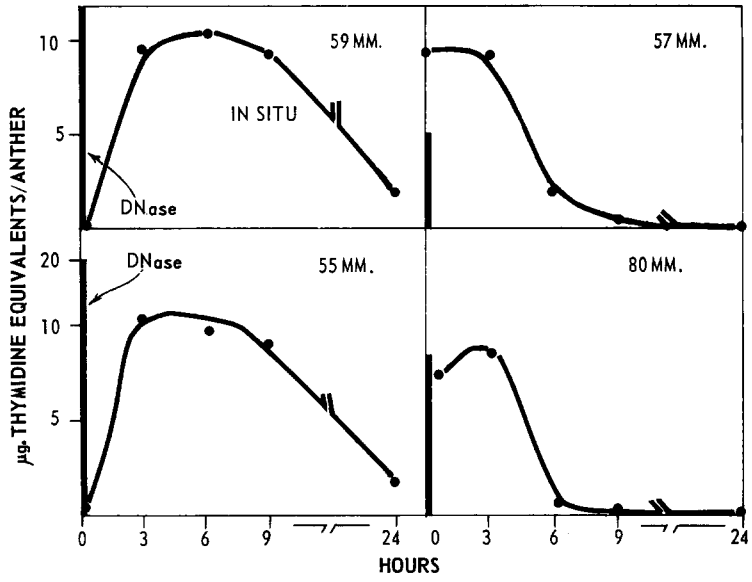

FIGURE 2

Relationship of DNase to cyclic production of deoxyribosides by *in situ* anthers. Buds were attached to plant during experiment (see text). Vertical lines represent initial DNase activity expressed as micrograms thymidine equivalents produced per homogenized anther in one-half hour at 25°C. Dots represent deoxyriboside content of *in situ* anthers as measured in 70 per cent methanolic extracts. "59 MM.," etc., is bud length in mm.

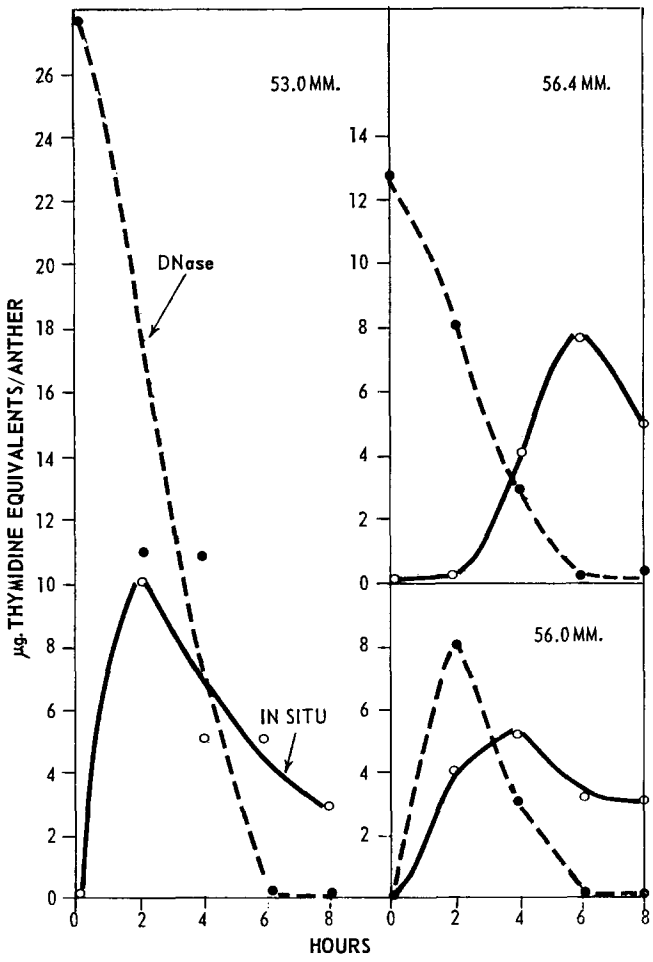

FIGURE 3

Periodicity in DNase activity and deoxyriboside appearance. "*In situ*" refers to deoxyribosides. Other representations as in Fig. 2.

been encountered at bud lengths of 80 to 90 mm. in which DNase activity rose during the course of the experiment but no deoxyribosidic pool could be detected. At these bud lengths too, however, if a pool did appear, it was preceded by DNase.

The point of greatest interest to these studies was the cyclical nature of DNase activity. Its duration, under conditions of growth as close to normality as possible, is of the order of 6 hours. This interval is brief compared with the 25 days covering the growth of microspores from the tetrad stage to postmitotic DNA synthesis, and even briefer compared with the life span of the wall cells. Although we have no knowledge of the molecular basis for the fluctuations in enzyme activity, it would be difficult to classify them other than as inductions of enzymatic activity. We have mixed suspensions of active and inactive anthers, but found the effects to be additive. Thus no evidence has been found for the existence of an inhibitory agent. Whether such induction represents a biosynthesis at the molecular level or transformation at a more complex one is incidental to the point that one of the ways in which cell development is governed is by a comparatively brief cyclical activation of enzymes. We have strong reason to suppose that DNase is not alone in this category. Our current studies on phosphorylation of the deoxyriboside pool (Hotta and Stern) indicate a similar pattern in phosphorylation. Evidence suggestive of periodic enzyme activity has also been found by Lark (2) in his studies of synchronously growing bacteria.

The locus and nature of the substrate which yields deoxyriboside remains an open question. Thus far we have been unable to identify the polydeoxyribonucleotide material which would appear to be the source of deoxyribosides. Superficially the DNA of the degenerating tapetum would appear to be the substrate but recent radioautographic studies of S. Takats (personal communication) indicate that labelled tapetal DNA does not transfer its label to the microspore chromosomes. Indeed, over the 8 to 12 hour period studied it has been found virtually impossible to draw up any equation between the appearance of soluble deoxyriboside and disappearance of DNA in the anther. The total deoxyriboside appearing *in situ* is not much greater in amount than the variations in DNA content between individual anthers.

CONCLUSIONS

The principal conclusion to be drawn from these studies is that periodic induction of enzyme activity over a brief interval in the life span of a cell is a mechanism for its morphogenetic development. The particular enzyme or process here studied is probably beside the point. The enzymatic mechanisms supporting the process may vary and the process itself may be continuous or discrete depending upon the cell type concerned. But insofar as sharply defined events occur during cell development, mitosis furnishing a striking example, then such events may well follow equally defined transformations in metabolic machinery. The microsporė, because of its relatively slow development, probably makes possible a refined study of its temporal properties which would be difficult in other cells. Even so, we have already pointed out (3) that broad cycles of metabolic change occur in the anther which bear only a secondary relationship to the briefly enduring structural changes which the cells undergo. The contrast is not that of relative importance but that of immediacy of effect. Much has been written about comparative increases and decreases in enzymatic activities of proliferating cells in normal and malignant tissues (1), but from our studies of anther development, it may well be that such changes are of the secondary type. What agent regulates the sharp cyclical enzymatic changes would appear to be a most significant question.

Contribution No. 118, Plant Research Institute, Ottawa.
Received for publication, September 4, 1960.

BIBLIOGRAPHY

1. STERN, H., *Tr. New York Acad. Sc.*, 1960, in press.
2. LARK, K. G., *Biochim. et biophysica. acta*, 1960, in press.
3. HOTTA, Y., and STERN, H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 279.
4. CORBY, N. S., KENNER, H. W., and TODD, A. R., *J. Chem. Soc.*, 1952, Part II, 1239.
5. SINSHEIMER, R. L., and KOERNER, J. F., *J. Biol. Chem.*, 1952, **198**, 293.
6. ALLFREY, V. G., and MIRSKY, A. E., *J. Gen. Physiol.*, 1952, **36**, 227.
7. HOFF-JØRGENSEN, E., *Biochem. J.*, 1951, **50**, 400.