DEAMINATION OF DEOXYCYTIDINE AND 5-METHYLDEOXYCYTIDINE IN DEVELOPING ANTHERS OF *LILIUM LONGIFLORUM* (VAR. CROFT)

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ABSTRACT

A study has been made of developmental variations in anthers of *Lilium longiflorum* with respect to the deamination of deoxycytidine and 5-methyldeoxycytidine. Although a periodicity of enzymatic activities surrounding the mitotic cycle has been found, the over-all evidence is against the significance of such periodicity to chromosome duplication. On the basis of evidence obtained a distinction has been drawn between enzyme systems functioning in a broad supporting role and those immediately tied to morphogenetic events.

INTRODUCTION

Commonly, metabolic transformations of deoxyribosidic compounds are considered to be associated with chromosome duplication. This is so because of the unique composition of deoxyribonucleic acid (DNA) molecules. That the capacity of cells to mediate such transformations is not directly linked to DNA synthesis has been demonstrated in the case of deoxycytidine (CDR) deaminase of *Escherichia coli* (1). However, studies of variations in cellular content of enzymes deaminating the phosphorylated derivatives of CDR and 5-methyldeoxycytidine (MCDR) have indicated a relationship between such variations and DNA synthesis (2, 3). In view of the fact that the initial substrate pool for DNA synthesis in anthers consists of deoxyribosides rather than deoxyribotides (4), it appears possible that a parallel may exist in function of deaminase activities between the animal tissues which act on phosphorylated derivatives and anthers which act on the non-phosphorylated forms.

This report arises from the observation that growing anthers of *Lilium longiflorum* are capable of deaminating CDR and MCDR to deoxyuridine (UDR) and thymidine (TDR) respectively. This capacity varies cyclically with development, intervals of peak activity surrounding those of DNA synthesis. Our studies have therefore been directed towards determining the extent to which such deaminations serve the requirements for DNA synthesis. The substance of our findings is that the deamination of CDR and MCDR has little, if any, direct bearing on DNA synthesis in the developing microspores of the anther.

METHODS

(*a*) *Preparation of Tissues:* "Intact" anthers, homogenates of these, and microspore suspensions were assayed for enzymatic activities. Anthers designated as "intact" were actually exposed at one end by a transverse cut about 1 to 2 mm. from the base and immersed in the desired solution to a depth of 1 to 2 mm.; no detectable absorption of deoxyriboside was observed over 18 hours if truly intact anthers were thus immersed. Microspores were obtained in the following way: glass rods were drawn to a diameter slightly smaller than that of the anther locule, and the tips of the rods fire-polished. Both ends of the anther were removed by transverse cuts, and a rod pushed through each of the locules. Below bud

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lengths of about 58 mm. the microspores and associated material emerged as a paste-like cylinder. In longer buds there is a progressive dehydration of the locule interior and weakening of the wall seam to a point where the microspores form a loose, moist aggregate and the anther wall is detached along one edge. During this interval microspores were removed by means of a scoop, which was simply a 1 to 2 mm. disc pressed at the end of a fine glass rod. About 80 to 90 per cent of the microspores are removed in this way without appreciable injury to the wall tissue. Depending upon the stage of anther development, variable amounts of other materials are removed with the microspores. For purification, they were suspended and lightly centrifuged in 0.35 M sucrose.

(*b*) *Enzyme Assays:* Six anthers of 1 bud were homogenized in 2 ml. of 0.1 M tris buffer (pH 7.6). Then, 0.1 ml. of CDR and MCDR (5 mg./ml.) was added to 0.2 ml. of the suspension and the mixture incubated at 25°C. for 30 minutes. The reaction was stopped with 0.3 ml. absolute methanol and the mixture heated to 60°C. for 2 minutes. After cooling in an ice bath, it was centrifuged and 0.2 ml. of the supernatant fluid was streaked on Whatman No. 1 filter paper. Two similar solvent systems were used to differentiate the reaction products by the descending technique: *n*-butanol-water (86:14 by volume) according to Markham and Smith (5), and a mixture of *n*-butanol and 0.1 M or 0.05 M ammonium formate (pH 9.0). The latter was prepared by adding to 10 ml. of the ammonium formate enough *n*-butanol (approximately 68 ml.) to bring all the components into solution. A layer of *n* butanol was placed at the bottom of the tank and two Petri dishes containing ammonium formate placed in the butanol. This solvent system, although slower in movement than butanol-water, differentiates MCDR from CDR; in general, it appears to be a better solvent system for separating deoxyribosides. Reaction products were located by means of an ultraviolet lamp. In most cases, areas of interest were eluted with 0.1 N HCl and the absorbency of the eluates read at 262 and 280 mμ. for CDR to UDR transformations, and at 267, and 290 mμ for MCDR to TDR transformations. As a check on this method, and also to increase sensitivity of detection, chromatograms were cut into strips of 0.05 R_f intervals, eluted with water, and assayed microbiologically for deoxyriboside content (6).

"Intact" anthers were immersed in 0.15 to 0.2 ml. of a solution containing 100 to 150 μg. of CDR or MCDR and buffered by 0.1 M phosphate or tris (pH 7.6). The reaction was stopped with 0.3 to 0.4 ml. of absolute methanol plus enough 70 per cent methanol to bring the fluid volume to 1.0 ml. and the anther was homogenized in this solution. After centrifugation, 0.8 ml. of the supernate was removed and reduced to about 0.2 ml. in a vacuum oven (40°C.). The concentrate was streaked on paper and treated in the manner described above.

Microspores were incubated in 0.1 ml. of deoxyriboside solution. After stopping the reaction in a final volume of 0.5 ml. 70 per cent methanol, the procedure followed was like the one for intact anthers.

C^{14}-CDR was prepared by incubating C^{14}-cytosine with non-radioactive TDR in the presence of an aqueous extract of *Lactobacillus helveticus* (ATCC 10386) (7). The reaction products were separated on paper in an isopropanol-HCl system (8).

RESULTS

Aqueous extracts of anthers over a wide range of developmental stages can deaminate CDR and MCDR to UDR and TDR respectively. The pH optimum of the reaction lies in the vicinity of 7.5–7.7, activities falling off sharply below a pH of 7.0 and above that of 8.0. The enzymatic content of the tissue is considerable; the extract from a single anther produces 1 μmole of UDR in 1 hour at 25°C. We have used such extracts to prepare C^{14}-UDR from C^{14}-CDR because the UDR formed undergoes very little, if any change. Cytidine is also deaminated by the anther but at 1/3 to 1/2 the rate of the deoxyriboside. No appreciable side reactions have been detected so that recoveries from the chromatogram of CDR + UDR or MCDR + TDR have been of the order of 90 per cent. The identity of the products was established by their position on the chromatogram, their ultraviolet absorption spectrum, and their growth-promoting activity with *Lactobacillus acidophilus*.

The phosphorylated derivatives CDRP, and presumably MCDRP, are not deaminated in this system (Table I). With tris buffer, homogenates of anthers convert CDRP to CDR and UDR. With phosphate buffer, however, CDRP is quantitatively recovered because of phosphate inhibition. Lowering of the pH from 7.6 to 6.6 (acetate buffer) does not affect dephosphorylation but reduces deamination. These effects are difficult to demonstrate with "intact" anthers because of the limited changes which external solutes produce in the internal milieu of the anther. However, if "intact" anthers are cultured for 15 hours in the presence of an adequate concentration of CDR or MCDR (see Methods), deamination is readily demonstrated for the absorbed deoxyriboside. In one such experiment, for example, 54 per cent of the 150 μg. CDR supplied was converted to UDR. The behavior of the anther is thus markedly different from that of embryonic and neoplastic

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TABLE I

Conversion of CDRP in Anther Homogenates

Bud Length	pH	Buffer	CDRP added	CDRP found	CDR	UDR
mm.						
48.4	7.5	phosphate	4.9	4.9	—	—
48.4	6.6	acetate	2.3	0.15	1.9	—
50.4	7.6	tris	2.1	0.8	0.6	0.6

Values are for micromoles/anther after 30 minutes at 25°C. Conditions as described under Methods. — signifies less than 0.1 μmole.

TABLE II

Conversion of C^{14}-CDR and C^{14}-UDR in Young Anthers

Bud length	Substrate	CDR	UDR	TDR	Remainder
11.2	C^{14}-CDR (1000)	606	324	65	44
11.5	C^{14}-UDR (1600)	—	1600	4	—
14.5	C^{14}-CDR (1000)	700	200	34	46
	C^{14}-UDR (1860)	—	1800	—	—

Values are for total counts/minute as determined after elution of spots from paper chromatograms. "Remainder" represents counts found among phosphorylated derivatives and methanol-insoluble residue (see Table IV). Conditions described in text. No potential methyl-group donors were used in these experiments.

tissues of rat liver (2) and of developing sea urchin eggs (3) in which deamination occurs only via the phosphorylated derivatives.

To pursue the biochemical facet of the problem a little further, we considered the possible fate of UDR. *In vivo*, there appears to be no marked accumulation of UDR in the deoxyribosidic pools, a situation which could be attributed either to inaccessibility of the enzyme to CDR (evidence for which will be considered later) or to transformation of the UDR found. Clearly, the usefulness of UDR as a precursor for DNA depends upon its convertibility to TDR. For this reason, folic acid, tetrahydrofolate, formaldehyde, serine, and adenosine triphosphate were used in various combinations under aerobic and anaerobic conditions, but no significant amounts of TDR were detected with the homogenates used. If anthers at the interval of tapetal DNA synthesis were incubated with approximately 2 μg. of C^{14}-CDR or C^{14}-UDR, a little C^{14}-TDR was formed in the presence of labelled CDR, and far less, if any, was formed in the presence of labelled UDR, indicating the improbability of UDR being an intermediate in the CDR to TDR conversion (Table II). This point is underlined by the observation that the capacity of a particular anther to form TDR is unrelated to its deaminative capacity.

The principal reason for not pursuing these biochemical studies lies in our view that the cyclical pattern in deaminative activity is of a type quite different from that encountered in systems directly concerned with DNA synthesis. The variations in deaminase activities are plotted against bud length in Fig. 1. Two peaks of activity appear on wither side of microspore mitosis, and these correspond roughly to the pre- and postmitotic intervals of DNA synthesis. It is clear, however, that the sharp changes which characterize the formation of deoxyribosidic pools (9) are not matched by the activities of this enzyme system. Even within a brief interval of development (1 day of growth) the presence of appreciable amounts of deoxyribosides is a nearly all-or-none phenomenon: anthers from identical bud lengths may or may not contain the soluble pool. By contrast, all anthers selected from a much broader interval of development have deaminative activities of a similar order of magnitude. The level of deaminase is not at all tied to the level of deoxyriboside substrate. The broad cycles of change in deaminative activity is much the same as that observed in the cases of

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TABLE III

Localization of CDR Deaminase in Lily Anthers

Bud length, *mm.*	51.0	54.0	59.0	75.0	80.5
Microspore region	0.01	0.05	0.04	0.01	0.01
Wall tissues	0.47	0.37	0.53	0.6	1.0

Values are for micromoles of UDR produced per anther in 1 hour. Conditions as described under Methods.

glutathione, ascorbic acid, phosphoglyceraldehyde dehydrogenase, and aldolase (9).

Such cycles need to be compared with those related to deoxyriboside production and transformation currently being studied. The latter are brief in duration (4 to 8 hours), nearly all-or-none in their manifestation, and are closely tied in time to specific mitotic events within the micropores (Stern,[1] Hotta and Stern[2]). The contrast at once suggests that the biochemical changes accompanying anther development fall into two classes: those which function in a broad supporting role and those which are immediately involved in the unfolding sequence of biochemical events constituting the chemical facet of mitosis proper.

A pointer to the probable indifference of CDR and MCDR deaminase to the sequence of events adjacent to or within the microspores is provided by the distribution of the enzyme system in the anther. Half or more of the deoxyriboside pool, when present, is found in the region of the microspores, and we presume that the remainder is

[1] Stern, H., data to be published.

[2] Hotta, Y., and Stern, H., unpublished data.

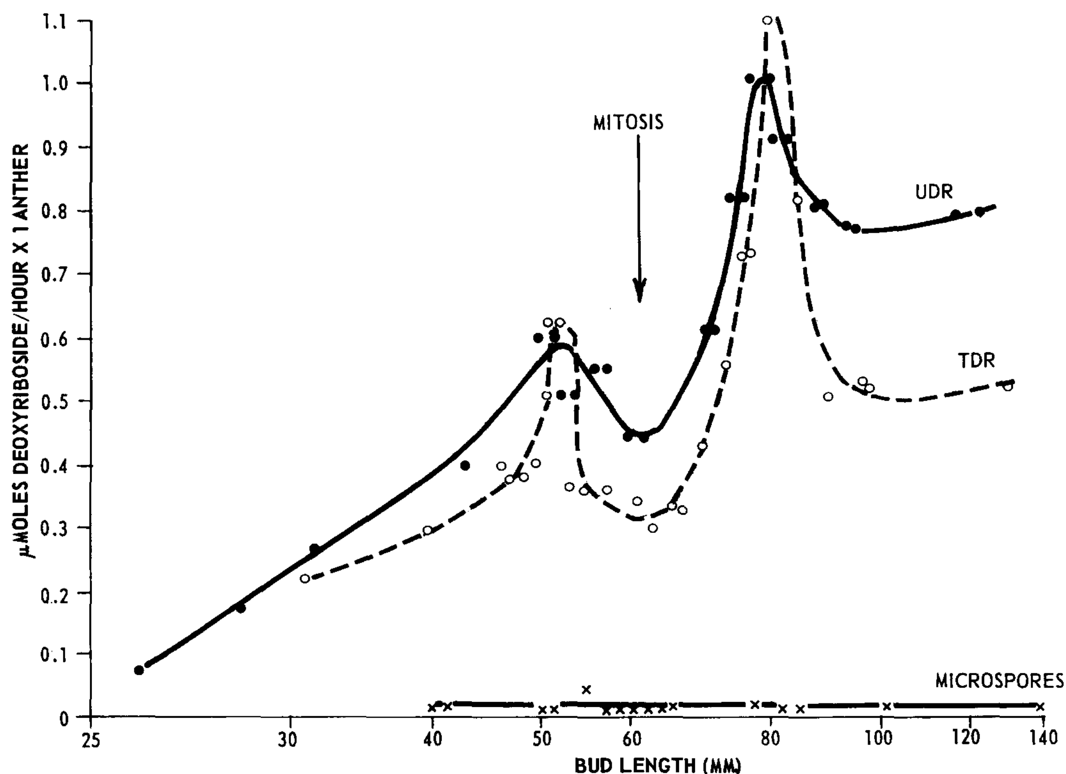

FIGURE 1

Deamination of CDR and MCDR in developing anthers of *Lilium longiflorum*. Solid line represents CDR deaminase and dotted line, MCDR deaminase. Dots and circles are for homogenates of whole anthers incubated at 25°C. "Microspores" refers to activities of the *unwashed fraction*.

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TABLE IV

Conversion of C^{14}-CDR in Lily Anther

Bud length, 95 mm.

Tissue	CDR added	CDR	UDR	TDR	Deoxynu-cleotides	Acid-solubles	Acid-insolubles
Wall	800	122	633	33	54	8	—
Microspores	800	191	251	—	378	24	4
Washed microspores	800	343	17	—	401	30	7

Values are for total counts/minute as under Table II. Conditions of incubation in text. Deoxynucleotides are those methanol-soluble products which remained at the origin in a butanol-water chromatogram (such material has since been identified by the use of an isobutyric acid-ammonium hydroxide system). Acid-solubles are components extracted with 5 per cent trichloracetic acid from the methanol-insoluble residue. Acid-insolubles are components subsequently extracted from that same residue with 1.0 N perchloric acid at 70°C.

probably found in the adjacent region of the anther not removed by the technique used (4). An opposite situation prevails with respect to deaminase; only a small proportion, if any, of total activity is found in the microspore region and none in the microspores themselves (Table III). "Intact" anthers which have been cultured for 15 hours in the presence of 100 μg. of CDR, roughly 50 times their normal peak complement of this compound, store most of the UDR formed in the wall tissue of the anther. Thus, in anthers which had been removed from 61.0 and 80.5 mm. buds, the first group formed 40 μg. of UDR within each anther, 8 μg. of which was found in the microspore region; the second group formed 37 μg. of UDR of which 5 μg. was in the microspore region. There appears to be no strong tendency for a diffusion of deoxyriboside towards the microspores; indeed, it is most probable, because of the anatomy of the anther, that CDR diffused into the wall tissue via the microspore region. On the whole, the evidence points to a physical separation of deaminase from the normal deoxyriboside pool and it is difficult to see how, under such circumstance, the enzyme system could appreciably influence the composition of that pool which has a direct role in DNA synthesis.

That enzyme systems closely associated with DNA syntheses have a markedly different distribution from CDR deaminase is illustrated by one experiment of a series currently being pursued. Wall tissue, unwashed, and washed microspores of anthers containing a phosphorylating system were separately incubated with C^{14}-CDR. This particular experiment has been selected because of the unusually high deaminase activity found in the microspore region. From the results summarized in Table IV it may be seen that whereas the microspore region is a set of substantial phosphorylative activity and low deamination, the wall tissue (contaminated though it is with microspores) is a seat of low phosphorylation and high deamination. Furthermore, washed microspores lose their capacity to deaminate but not their capacity to phosphorylate.

CONCLUSIONS

The principal feature of these studies has been the demonstration that some enzyme may mediate deoxyriboside transformations and yet not be directly tied to the sequence of events leading to chromosome duplication. Although a cyclical pattern of change in such enzymatic activities exists in temporal association with cycles of DNA synthesis, the apparent relationship between them turns out to be of a superficial nature. Anther development, like all organ development, is a morphogenetic process in which non-sporogenous and sporogenous tissues are participants. Although there may be a concurrence of cyclical metabolic patterns in the anther with the mitotic cycle in the microspores, one must nevertheless distinguish between supporting and morphogenetic metabolic processes. The former extend over a relatively broad interval of time and presumably support general growth requirements. The latter are brief in duration and are immediately associated with distinctive biosynthetic events occurring sequentially in the life history of a cell.

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