RADIOAUTOGRAPHIC LOCALIZATION OF DEOXYTHYMIDINE TRIPHOSPHATE IN *TRADESCANTIA* POLLEN GRAINS DURING DNA SYNTHESIS

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ABSTRACT

Tradescantia pollen grains, isolated during the period of DNA synthesis in the generative cell, accumulate deoxythymidine triphosphate (dTTP)-^{3}H after incubation with thymidine-^{3}H in the presence of millimolar deoxyadenosine. Most of this dTTP-^{3}H was found to resist extraction by the fixative, cold ethanol-acetic acid, and its location was investigated by radioautography with thin, dry emulsion. Substantial binding of dTTP-^{3}H occurred as an artifact; but when nuclei were isolated from the fixed pollen grains by sonication, it was found that they were differentially labeled: generative nuclei contained dTTP-^{3}H, vegetative nuclei did not. This observation is discussed and is interpreted as evidence supporting the idea that thymidine is phosphorylated only in the generative cell of the pollen grain.

INTRODUCTION

Pollen grains of *Tradescantia* possess a mechanism for controlling DNA metabolism which restricts the synthesis of DNA to only one (the generative cell) of the two sister cells in the pollen grain. In this respect these pollen grains are typical of angiosperms. Deoxyadenosine interferes with the incorporation of thymidine into DNA and causes the accumulation of dTTP[1] in *Tradescantia* pollen grains (Takats, 1967). On the basis of radioautographic evidence, it was reported (Takats, 1965 *a*) that in the presence of deoxyadenosine, dTTP was synthesized from TdR in both the generative cell and the vegetative cell. Since this observation suggested that both cells could phosphorylate deoxynucleosides and deoxynucleotides, it was thought that the mechanism normally preventing DNA synthesis in the vegetative cell did not involve a simple deficiency of deoxynucleotide synthesis. The present report considers in more detail the techniques and results of the earlier work, and it gives new results which show that the earlier report probably was erroneous. These results are consistent with the restriction of dTTP synthesis to the DNA synthetic cell, the generative cell.

[1] The following abbreviations are used: TdR, thymidine; dTMP, dTDP, dTTP, thymidine-5′-monophosphate, -diphosphate, and -triphosphate, respectively; AdR, 2′-deoxyadenosine; EA, absolute ethanol: glacial acetic acid (3 vol: 1 vol).

MATERIALS AND METHODS

Growth conditions for *Tradescantia* and the procedures for isolating and labeling whole pollen grains with radioactive TdR have been described (Takats, 1965 *b*, 1967). Freshly prepared, ice-cold ethanol: acetic acid (3:1 by volume) was used to fix the pollen grains.

Radioautographs were prepared in two ways: (*a*) by dipping air-dried Feulgen squashes of pollen

grains in Kodak NTB2 liquid emulsion; (*b*) by applying thin air-dried loops of Kodak NTB2 or NTB3 emulsion onto air-dried, unstained pollen grains on slides, according to the technique of Caro and van Tubergen (1962) as modified by Miller et al. (1964). Several variations on the latter technique were also tried and will be described in the Results section. Exposure of the radioautographs took place in the presence of Drierite; development was done at 18°C for 2 min in Dektol, followed by a 30 sec water rinse and by fixation for 3 min in acid-fixer (Kopriwa and Leblond, 1962). Washing after fixation was limited to 10 min, since prolonged washing produced air bubbles in the emulsions that were applied as dry loops. All radioautographs were air-dried and then mounted in Euparal before examination.

Cold 5% trichloroacetic acid extracts of labeled pollen grains, for chromatographic analysis of acid-soluble products following TdR-^{3}H labeling, were washed three times with ice-cold ether to remove trichloroacetic acid. The extracts were then lyophilized, dissolved in minimum volumes of water, and spotted on No. 1 or No. 4 Whatman filter paper together with the appropriate nonradioactive markers. Ascending development was carried out with one of three solvent systems: (*a*) *n*-butanol:acetone:acetic acid:ammonium hydroxide:water (Randerath, 1962); (*b*) isobutyric acid:ammonium hydroxide:water (Krebs and Hems, 1952); (*c*) *n*-butanol:water (Vischer and Chargaff, 1948). Paper strips were scanned with a Vanguard scanner (Vanguard Instrument Corp., Roosevelt, N.Y.); UV-absorbing spots were eluted with water and counted in a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). dTTP-^{3}H was obtained from Schwarz Bio Research, Inc., Orangeburg, N.Y.; its specific activity (measured) was 1 μc per 0.437 mμmole. Less than 10% was present as dTDP-^{3}H.

Sonication of pollen grains was carried out in an ice bath with a Branson Model 75 sonicator (Branson Instruments, Inc., Stamford, Conn.), with the microtip attachment.

RESULTS

Differential Extraction by Cold Ethanol-Acetic Acid (EA)

Table I shows that when cold EA was used to fix pollen grains which had been incubated with TdR-^{3}H in the presence of AdR, the EA differentially extracted nucleosides and nucleotides, removing nearly all the TdR-^{3}H but leaving behind most of the nucleotide derivatives. (Very little dTDP-^{3}H was actually found; it is reported here together with dTTP-^{3}H because it was not completely resolved from dTTP-^{3}H in the usual chromatograms.) Only about 1/5 of the dTTP-^{3}H was removed by EA; the remainder could be removed by cold 5% trichloroacetic acid, as is shown in Table I. This differential extraction made it possible, by radioautography, to look for dTTP-^{3}H directly in the cells of the fixed pollen grains.

TABLE I

Distribution of Soluble Radioactivity

Aliquot	Extract	dTTP + dTDP	dTMP	TdR	Total
		cpm	*cpm*	*cpm*	*cpm*
A	1st, cold EA	563	136	1094	1793
	2nd, cold trichloroacetic acid	2407	111	19	2536
		2970	247	1113	4329
B	cold trichloroacetic acid (directly)	3049	290	1029	4368

Pollen grains were incubated for 30 min with TdR-^{3}H (3 μc/ml, 0.50 mμmoles/ml) in the presence of 1 mM AdR. Extracts were analyzed by paper chromatography with Randerath's No. 4 solvent (Randerath, 1962). Recovery of counts in both aliquots was 85% of the total soluble radioactivity.

Nearly all the labeled material in the pollen grains that was soluble in cold EA came out in the first extraction. Repeated extraction removed only an insignificant additional amount of label (e.g. 95, 4, and 1% of the total EA-soluble labeled material was removed in three successive extractions of 18, 24, and 22 hr, respectively, at −20°C). The inability to continue extracting labeled material with cold EA was originally taken as evidence that the location of the residual dTTP-^{3}H in the

cells was not changed, since the dTTP-^{3}H would have to be solubilized to be redistributed. However, it was not possible to rule out redistribution and rebinding of nucleotide in the cells during the initial extraction (fixation). This point will be returned to in procedures *d* and *e* below.

Procedures to Localize the Residual dTTP-^{3}H

(*a*) When cold EA-fixed whole pollen grains, which had been previously incubated with TdR-^{3}H in the presence of AdR, were washed with absolute ethanol, allowed to air-dry, and covered with liquid NTB2 emulsion, no radioactive label was found. Label was detected only when such pollen grains were covered with very thin air-dried loops of NTB2 or NTB3 (see Materials and Methods). Both cells of the pollen grain were then observed to be labeled, with no concentration of label in either nucleus (Fig. 1). Label was completely absent if the pollen grains were extracted with cold 5% trichloroacetic acid for 15 min.

(*b*) Considerable spreading of label away from the pollen grains was a typical feature of the radioautographs prepared by applying dry loops. This spreading was apparently due to leaching of dTTP-^{3}H into the emulsion which contains some water and hence acts as an aqueous extractant. A number of attempts were made to reduce this spread of label: (*1*) evaporation of a carbon film onto the dried pollen grains on slides before dry-looping at room temperature, in order to interpose a thin hydrophobic layer of carbon between the pollen grains and the aqueous emulsion (Rossi and Baic, 1964); (*2*) treating the pollen grains with Ca^{++} in cold ethanol before drying them down on slides, in order to precipitate the dTTP-^{3}H *in situ* as the calcium salt and thus reduce diffusion (Pontis and Blumson, 1958); (*3*) freezing the dry-looped emulsion on slides and then applying pollen grains in a xylene suspension onto this emulsion at −20°C, in the dark in a cryostat, with subsequent exposure at −100°C (Appleton, 1964); (*4*) exposure at −100°C after routine application of dry loops at room temperature onto the dried pollen grains on slides. The last two procedures were also intended to reduce diffusion. None of these procedures was successful in appreciably reducing the spread of label, although they were not tested exhaustively. No explanation can be given for the inability of the carbon film to reduce spreading, unless the carbon films were not thick enough. The carbon films were applied at different thicknesses by varying the time of carbon arcing in a Kinney vacuum evaporator (Kinney Vacuum Div., Boston, Mass.); actual thicknesses were not determined.

(*c*) Evidence that dTTP-^{3}H was not superfi-

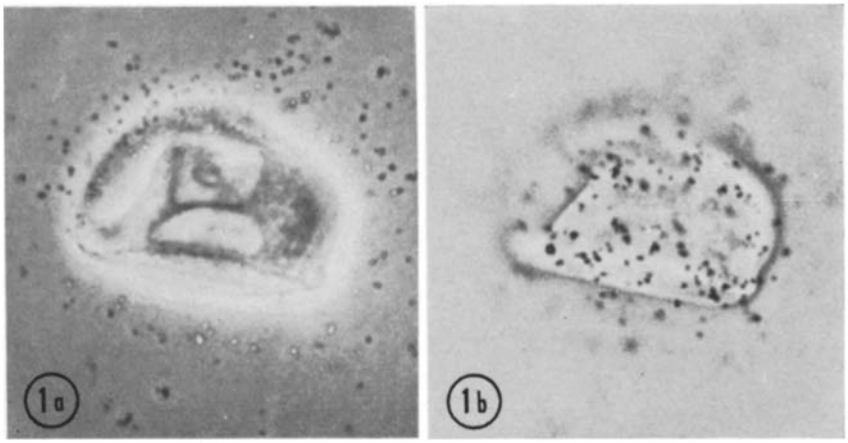

FIGURE 1 Radioautograph of *Tradescantia* pollen grain isolated during the normal period of DNA synthesis, incubated for 30 min with TdR-^{3}H in the presence of 1 mM AdR, fixed with cold EA, and air-dried on the slide. 1*a* shows the unstained phase-contrast image at the level of the pollen grain; the generative nucleus is at the bottom, the vegetative nucleus is at the top (with single large nucleolus). The generative cell is only slightly larger than the generative nucleus; most of the volume of the pollen grain consists of the vegetative cell with large vacuoles. 1*b* shows the distribution of silver grains in the emulsion over the pollen grain. Approximately, × 1100.

cially bound to the walls of the pollen grains was obtained by sonicating cold EA-fixed pollen grains. Breakage was monitored microscopically; the sonicated preparations contained a variety of wall fragments, intact and broken pollen grains, intact and broken cells, and free nuclei. Radioautographs of these preparations, stained with Giemsa through the emulsion (Gude et al., 1955), showed that the label was present only in intact pollen grains, in fragments of pollen grains or cells, and in free nuclei. Wall fragments were not labeled.

(*d*) Label in nuclei was detected with much greater efficiency when the nuclei were freed from the overlying cytoplasm and wall of the pollen grain, which otherwise absorbed most of the electrons emitted. For example, a sample of pollen grains (after incubation with TdR-^{3}H and AdR, fixation with cold EA, and extraction with cold trichloroacetic acid) gave 5% of the pollen grains with labeled nuclei, vs. 50% isolated labeled nuclei, in radioautographs exposed for the same length of time. Since each pollen grain contains two nuclei, only one of which has labeled DNA, the expectation is 2.5% isolated labeled nuclei. The data are biased because sonication causes a preferential destruction of vegetative nuclei, but even with a complete loss of vegetative nuclei the expectation is only 5%.

If it is assumed that dTTP-^{3}H in the nuclei was not redistributed during fixation, then labeled nuclei of both types should be present only if both cells were labeled. With this assumption, if nuclei are unlabeled, then dTTP-^{3}H is not made in them. In order to obtain data on this point, the following procedure was carried out: dry-looped radioautographs were prepared, and the unstained nuclei were scored as labeled or unlabeled. The positions of the nuclei were recorded photographically, and the silver grains were then removed from the emulsion with the use of Farmer's reducer. The emulsion was coated with collodion, and the preparation was hydrolyzed and stained by the Feulgen procedure. Nuclei previously scored for the presence or absence of label were then relocated and scored as vegetative or generative, based on size and nucleolar morphology. The results are shown in Table II. Of the generative nuclei, 65% were labeled. Only 4% of the vegetative nuclei were labeled, and the single nucleus which this represents was questionably classified. The probability

TABLE II

Radioactivity in Sonication-Released Nuclei

	No. of Nuclei in Radioautographs			Labeled
	Labeled	Unlabeled	Total	
				%
Generative	163	89	252	65
Vegetative	(1)	26	27	(4)

See text (Results, Procedures to Localize the Residual dTTP-^{3}H (*d*)) for description. Pollen grains were incubated with TdR-^{3}H in the presence of AdR, then fixed, and sonicated to release nuclei; radioautographs were then prepared.

of obtaining this many unlabeled vegetative nuclei by preferential destruction of the labeled nuclei is very small.

The dTTP-^{3}H in these nuclei was peculiarly resistant to extraction by cold 5% trichloroacetic acid. About one-half to two-thirds of the label was retained after 20-min extraction by trichloroacetic acid at 4°C. Nevertheless, all the label was removed when the nuclei were hydrolyzed for the Feulgen reaction. The latter extraction, 8 min in 1 N HCl at 60°C, should not remove pyrimidines from DNA. Hence the label is probably not in DNA, but rather in a form which is more tightly bound than the usual unpolymerized nucleotides. The possibility that the label is actually in nascent DNA is considered in the Discussion.

A correspondence was observed between the frequency of labeled nuclei from samples incubated with AdR (59%) and the frequency of labeled nuclei from controls incubated without AdR (51%). This observation suggests that only those nuclei engaged in DNA synthesis accumulate dTTP-^{3}H. There are not enough data, however, to establish this point firmly.

(*e*) The presence of label in the intact pollen grains was originally taken to mean that the dTTP-^{3}H was made in the pollen grains and was present in both cells. Therefore, if dTTP-^{3}H were added exogenously with the fixative (EA), the result should be unlabeled pollen grains. Experiments were carried out in the following way to check this. Approximations were made to the amount of dTTP-^{3}H present in whole pollen grains following the usual incubation with TdR-^{3}H in the presence of AdR, and amounts of authentic dTTP-^{3}H in this range were added to EA. Pollen grains were incubated in the absence of

TdR-^{3}H and were then fixed with cold EA containing dTTP-^{3}H. Radioautographs were prepared in the usual way, by dry-looping NTB2 onto the dried pollen grains on slides.

The result in all cases was that pollen grains were uniformly labeled. The radioautographs were, in fact, indistinguishable from the radioautographs prepared from pollen grains incubated with TdR-^{3}H. The general conclusion from these experiments is that the presence of label in both cells of pollen grains incubated with TdR-^{3}H is not a necessary indication that dTTP-^{3}H is made in both cells. Rather, the dTTP-^{3}H could be redistributed by EA and bound artifactually.

In order to examine the differential nuclear labeling described in Procedures to Localize the Residual dTTP-^{3}H (*d*) above, the nuclei from artifactually-labeled pollen grains were obtained by sonication and scored in radioautographs for the presence or absence of label. The results were variable, but in several experiments 100% of the nuclei was labeled. Thus, both generative and vegetative nuclei bound dTTP-^{3}H as an artifact. Although sufficient data are not available, there is basis for thinking that the variation in the frequency of labeling was due to variation in the amount of dTTP-^{3}H supplied. The results in Table II may then be interpreted if the assumption is made that the concentration-dependence of labeling is not markedly different for the two types of nuclei. Since it is clear that all the generative and vegetative nuclei can be labeled artifactually at the concentration of dTTP-^{3}H used, it is unreasonable to expect that vegetative nuclei from pollen grains that made dTTP-^{3}H would be entirely unlabeled at a concentration that gave 65% labeled generative nuclei. The conclusion, then, is that nuclei from pollen grains incubated with TdR-^{3}H were truly differentially labeled.

An additional difference was observed between the artifactually-labeled nuclei and the nuclei that made dTTP-^{3}H from TdR-^{3}H. The former lost all their label upon extraction with cold 5% trichloroacetic acid, whereas the latter were partially resistant to extraction. This may be taken as further evidence in support of the reality of differential nuclear labeling from pollen grains incubated with TdR-^{3}H.

DISCUSSION

Several previous studies of the uptake and incorporation of radioactive TdR in a variety of materials (Appleton, 1964; Crathorn and Shooter, 1960; Feinendegen and Bond, 1962; Stone et al., 1965) have shown by radioautography that the soluble derivatives of TdR may be retained in cells if suitable procedures are used. Crathorn and Shooter (1960) presented data which are probably most pertinent to the present results: after supplying TdR-^{3}H to ascites cells, they found that the radioactivity detected in radioautographs exceeded the radioactivity in the DNA extracted from the same cells. This excess radioactivity was attributed to TdR-^{3}H and its nonpolymerized derivatives in the cells, which resisted fixation by EA.

The present results with *Tradescantia* pollen grains show that cold EA fixation removes most of the nonpolymerized derivatives, leaving dTTP-^{3}H. The reason that cold trichloroacetic acid fails to remove all the label from sonication-released nuclei is not understood. It is possible that some of the label thought to be dTTP-^{3}H is actually nascent DNA-^{3}H. The results from direct scintillation counting of pollen grain extracts show that the ratio of acid-soluble to acid-insoluble counts is approximately 100:1 (Takats, 1967). Since the DNA was not isolated in these experiments, the acid-insoluble counts may include the same resistant label that is now found by radioautography in the isolated nuclei. Of the acid-soluble counts, about 70% is dTTP-^{3}H, and as is shown in Table I, about 80% of this remains in the pollen grains after fixation. Hence, the ratio of acid-soluble dTTP-^{3}H to total acid-insoluble ^{3}H in the fixed intact pollen grains is about 56:1. This finding means that, of the total label in fixed intact pollen grains, DNA-^{3}H contributes no more than about 2%. However, much more than 2% (at least 50%, by grain counts) of the label in isolated nuclei resists cold trichloroacetic acid extraction. Unless the nuclei contain only a small proportion of the dTTP-^{3}H in the pollen grain, these considerations make it unlikely that much of the label is DNA-^{3}H. In any case, since the labeled nuclei are all generative, the dTTP-^{3}H also must be restricted to the generative nuclei.

The results of the binding experiments reported in Procedures to Localize the Residual dTTP-^{3}H (*e*) suggest that, when pollen grains are supplied TdR-^{3}H, both nuclei should bind dTTP-^{3}H. The fact that isolated nuclei are not both labeled may be tentatively explained by assuming that

much less dTTP-^{3}H is available to the vegetative nuclei when dTTP-^{3}H is formed locally from TdR-^{3}H than when it is supplied exogenously in EA.

These results together suggest that dTTP-^{3}H is synthesized from TdR-^{3}H by the DNA synthetic cell, the generative cell, and that a small amount of dTTP-^{3}H is redistributed during fixation with cold EA. dTTP-^{3}H is bound by both cells in the pollen grain, principally in the cytoplasm. In intact pollen grains, cytoplasm is present between the nuclei and the emulsion, hence radioautographs show uniformly labeled pollen grains. Although the participation of the vegetative cell in dTTP synthesis cannot be excluded by the present results, the failure to find radioactive labeling in the vegetative nucleus, except as an artifact, is evidence against this. It is assumed, of course, that the vegetative cell is permeable to TdR in vivo. While there is no direct evidence to support this assumption, it is known (Takats, unpublished data) that radioactive cytidine and uridine rapidly label RNA in both cells in intact pollen grains.

AdR was used in the present study in order to eliminate labeling of DNA and to accumulate sufficient dTTP-^{3}H in the intact pollen grains to make visualization of dTTP-^{3}H easier. The assumption made is that AdR does not alter the ability of either cell to phosphorylate TdR, but that AdR blocks the incorporation of dTTP into DNA. Previous studies (Takats, 1967) showed that the initial net rate of uptake of TdR-^{3}H into intact pollen grains is unaltered by incubation with AdR. Pollen grains incubated with TdR-^{3}H in either the presence or absence of AdR show uniform labeling in dry-looped radioautographs. Nuclei isolated from pollen grains incubated in the absence of AdR have not been examined for differential labeling.

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