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Lack of Effect of Microtubule Poisons on the 93D or 93D-like Heat Shock Puffs in *Drosophila*

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In view of the specific inducibility of the 93D heat shock (HS) puff of *D. melanogaster* by colchicine [Lakhotia & Mukherjee, *Indian J Exp Biol*, 22 (1984) 67], salivary glands of different species of *Drosophila* have been exposed to several other microtubule (MT) poisons, viz. cold shock, chloral hydrate, diamide, podophyllotoxin, vinblastin, griseofulvin and nocodazole to examine the inducibility of 93D or 93D-like puff. It is observed that except cold shock, none of the other MT poisons induces the 93D or 93D-like puff. Podophyllotoxin, a competitive inhibitor of colchicine binding to microtubules, fails to counter the induction of the 93D puff by colchicine. Thus it appears that induction of the 93D puff by colchicine treatment is not due to its direct effects on microtubules.

The 93D or 93D-like heat shock puff is unique in being selectively inducible by several agents[1-8]. The functions of these loci in different species of *Drosophila* remain unclear. In this context, the observation that colchicine or colcemid can also specifically induce the 93D puff of *D. melanogaster*[8] is interesting. Colchicine action in inducing a specific heat lock puff was first reported in *D. virilis* by Gubenko and Baricheva[9]. They found that colchicine induces one of the heat shook puffs, 20 CD, in *D. virilis* which appears to be 93D-like in view of its inducibility by vit-B_6[4,6]. The most well known effect of colchicine or colcemid is on microtubules (MT) in the cell[10]. Thus the specific inducibility of 93D or 93D-like puff may suggest that this locus is in some way related to MT organization MTs are part of cytoskeleton system, and in recent years, several studies have revealed that heat shock and other agents that elicit heat shock response also exert some effects on the cytoskeleton system[11-13]. In view

examine their action on the heat shock puffs, especially the 93D locus.

Materials and Methods

Salivary glands from late third instar larvae of *D. melanogaster*, *D. ananassae*, *D. hydei* or *D. nasuta* were excised in Poels' salt solution as described earlier[1] and the following treatments were applied.

Cold shock—Freshly excised *D. melanogaster* salivary glands were transferred to 8 C for 60 min. Sister glands were kept as control and incubated at 24°C for 60 min. Some of the salivary glands were also cold shocked at 4°C for 1 or 2 hr. In another set of experiments, some of the cold shocked (8°C) salivary glands were allowed to recover at 24°C for 30 min.

Following the incubation period, the glands were fixed, stained and squashed in 50% acetic acid. The chromosome squashes were examined under phase-contrast optics for the activity of HS puffs, specially the 93D puff.

To study the effect of cold shock on RNA synthesis in salivary gland polytene chromosomes, cold shocked and control glands were pulse labelled with ^{3}H-uridine (300 μCi/ml; sp. act. 12.8 Ci/mM, BARC, Trombay) for 10 min at 8°C or at 24°C, respectively.

Treatment with different MT poisons—Freshly excised sister salivary glands from late third instar larvae of *D. melanogaster*, *D. ananassae*, *D. hydei* or *D. nasuta*, were incubated either in absence or presence of different MT poisons (see Table 1) for 45 min. Table 1 lists the specific MT poisons used, their solvents and solvent other than water (see Table 1), equal amount of the solvent was added to the controls. Following the treatments, glands of *D. melanogaster* were labelled with ^{3}H-uridine (300 μCi/ml) for 10 min at 24°C. The treated glands were labelled in presence of the particular MT poison. Salivary glands from other species were treated with some of the MT poisons (see Table 1) and after the treatment they were fixed, stained and squashed for examination by phase-contrast optics.

Combined colchicine and podophyllotoxin treatments—Podophyllotoxin is a potent MT poison whose cytological effects on tubulings appear very similar to those of colchicine and is also a competitive

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Table 1 — Summary of Different Treatments Applied to Salivary Glands of Larvae of *Drosophila* Species and Modes of Evaluation

Treatment	Conc. used	Conc. used	*Drosophila* sps and methods of examination
Cold shock (60 to 120 min at 4° and 8 C)	—	—	*D.melanogaster* (PS, ARG)
Colchicine	Water	5 μg/ml	*D.melanogaster* (PS, ARG) *D.hydei* (PS)
Chloral hydrate	do	1 mg/ml; 2 mg/ml	*D.melanogaster* (PS, ARG). *D.ananassae* (PS), *D. hydei* (PS), *D.nasuta* (PS)
Diamide	do	5 μg/ml; 20 μg/ml. 1 mg/ml	*D melanogaster* (PS, ARG) *D.hydei* (PS)
Podophyllotoxin	Alchohol	25 μg/ml; 100 μg/ml	*D.melanogaster* (ARG)
Vinblastin	Chloroform	1 μg/ml	do (ARG)
Griseofulvin	do	50 μg/ml	do (ARG)
Nocodazole	DMSO	5 μg/ml; 10 μg/ml; 100 μg/ml	do (ARG)

PS = puff size measurement; ARG = ^{3}H-uridine autoradiography

inhibitor of colchicine action[14]. To see the effect of podophyllotoxin on induction of the 93D puff of *D. melanogaster* by colchicine, salivary glands were first treated with podophyllotoxin (10 μg/ml) for 30 min and then incubated in medium having only colchicine (5 μg/ml) for 30 min. The control sister glands were incubated in podophyllotoxin-free medium for 30 min and then treated with colchicine (5 μg/ml) for 30 min at 24° C. After the incubation the control and the treated glands were labelled with ^{3}H-uridine (300 μCi/ml) for 10 min at 24° C in presence of colchicine.

In all experiments, the glands were fixed, stained and squashed immediately after labelling with ^{3}H-uridine. The chromosome preparations were processed for autoradiography in the usual manner[1].

The autoradiograms were scored for numbers of silver grains present on the HS puff sites (63BC, 67B, 87A, 87C, 93D and 95D) in different well spread nuclei. Silver grains on an autosomal segment (from 60A to 63A on 3L) a developmentally active puff at 75B or 90B and on nucleolus were also scored.

Results

The induced or uninduced activity of the HS puffs after different treatments is illustrated in Figs 1, 3, 5 and 7 while data on the activity of different HS puffs and the 3L segment (60A to 63A) following treatment with the various MT disrupting agents are presented as histograms in Figs 2, 4, 6 and 8. In each case, 25-45 nuclei from 5-10 salivary glands were examined.

Effect of cold shock (8° C)— When salivary glands are given a cold shock (8° C for 60 min), the 93D develops into a prominent puff (Figs 1b and 2). The rate of ^{3}H-uridine incorporation in all regions of polytene chromosome is significantly reduced after cold shock (see Figs 3 and 4a). The 93D region, however, appears well labelled (see Fig. 3b). Although the absolute value of the mean grain counts on the 93D puff in cold shocked glands is less (nearly half) than that in sister control glands maintained at 24° C (see Fig. 4a), in terms of relative activity (i.e. when compared with ^{3}H-uridine incorporation on 3L segment) the 93D puff is significantly more active after cold shock: the mean of 93D/3L grain ratio in control glands is 0.63, while in cold shocked glands it is 1.3 (Fig. 4b). Thus in view of the morphological puffing and its increased relative activity, cold shock appears to induce the 93D locus. Interestingly, the 63BC puff is also relatively more active in ^{3}H-uridine incorporation (see Fig. 4b) although morphologically it does not form a puff. None of the other heat shock puffs are induced by cold shock (Figs 1, 3 and 4b).

When cold shocked glands are allowed to recover at 24° C for 30 min, the 93D puff regresses but the other HS puffs are not induced (Figs 1C and 2). Cold shock at 4° C induces the 93D more slowly since after 1h at 4° C, the 93D remains uninduced while after 2hr it develops into a prominent puff (Fig. 2).

Effect of different MT poisons on heat shock puffs— Neither the 93D nor any other heat shock puff is induced, either morphologically or in ^{3}H-uridine incorporation by any of the MT poisons (chloral hydrate, diamide, podophyllotoxin, vinblastin, griseofulvin or nocodazole) tested in this study (Figs 5 to 8). As may be noted from the data on ^{3}H-uridine incorporation on the 3L-segment in Fig. 6, the water soluble MT poisons like chloral hydrate (1 mg/ml) and diamide (5 μg/ml) have an inhibitory effect on general chromosomal RNA synthesis (Fig. 6a). The chloroform or DMSO soluble compounds (griseofulvin, 50 μg/ml; vinblastin, 1 μg/ml; nocodazole, 10 μg/ml) do not have any effect on chromosomal

RNA synthesis (Figs 6b, c) although the ethanol soluble podophyllotoxin (50 μg/ml) inhibits chromosomal RNA synthesis, more so at the higher (100 μg/ml) concentration (Fig. 8a). As indicated in Table 1, some of the MT poisons have been used at higher concentration and for longer treatment times also. It is seen that chloral hydrate at 2 mg/ml inhibits RNA synthesis totally but does not induce the 93D puff even morphologically (data not presented). Similarly nocodazole treatment for 45 or 90 min at 100 μg/ml also does not induce the 93D puff (data not presented). It may be noted that the level of autoradiographic labelling varies even in control glands of different sets (Fig. 6). Therefore, for each

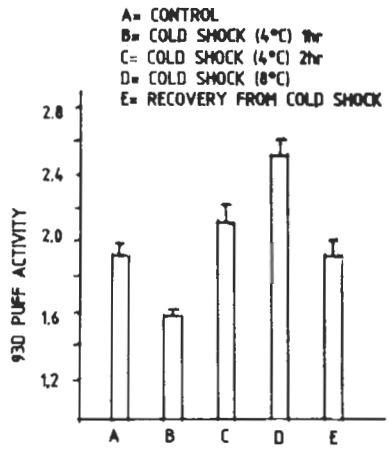

Fig. 2—Mean (±SE) 93D puff activity in salivary glands incubated at different temperatures. For each data point, 30-35 nuclei were observed from salivary glands of 5-6 larvae

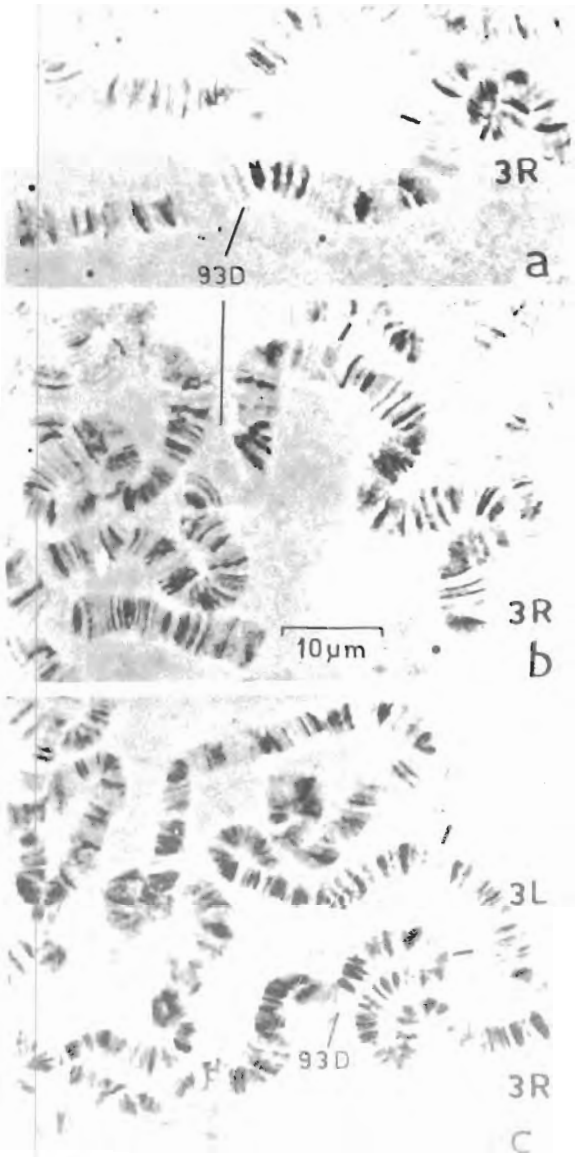

Fig. 1—Phase contrast photomicrographs of polytene chromosomes of *D. melanogaster* showing effect of cold shock (8 C) or recovery from cold shock: control (a), cold shock (8 C) for 60 min (b), and recovery for 30 min at 24 C from cold shock (c)

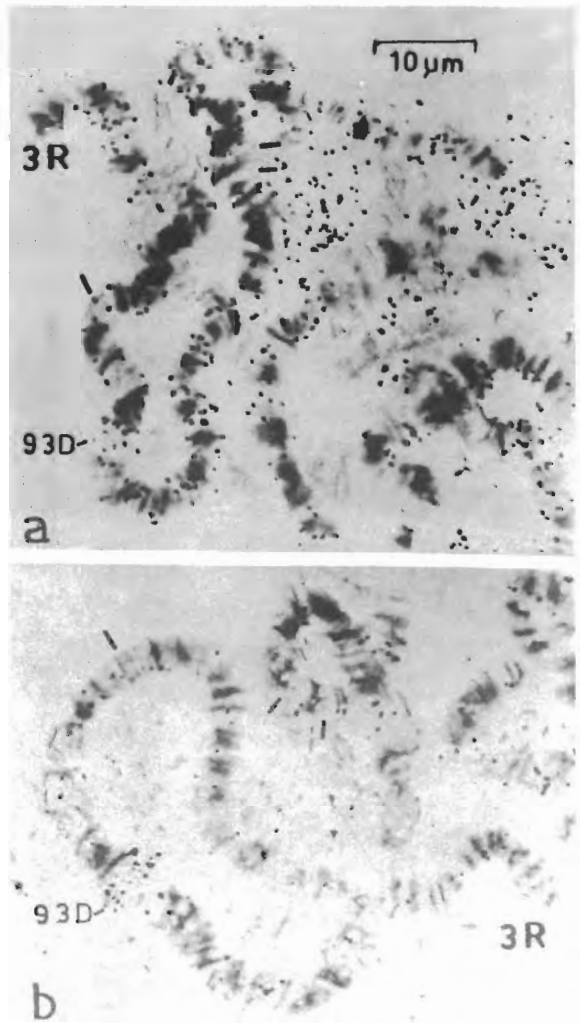

Fig. 3 Autoradiographs of salivary gland chromosomes of *D. melanogaster* showing effect of cold shock (8 C) on RNA synthesis: Control (a), cold shocked at 8 C for 60 min (b). Note the drastic inhibition of general RNA synthesis except at 93D in (b). Other heat shock loci (indicated by--) are nearly unlabelled

treated set, the sister glands have been used as control so that inter-gland variability is minimized.

Effect of some of the MT poisons like colchicine, chloral hydrate and diamide have been examined in other species of *Drosophila* also to see inducibility of 93D-like heat shock puffs (Table 1). It is seen that colchicine (100 μg/ml) specifically induces 2-48C puff in *D. hydei* but chloral hydrate (1 mg/ml) and diamide (5 μg/ml) are unable to induce this puff. Similarly in *D. nasuta*, chloral hydrate and diamide do not have any effect on the 2R-48A puff (data not presented).

Effect of podophyllotoxin treatment on induction of 93D by colchicine—As noted earlier, colchicine causes a specific induction of 93D puff in *D. melanogaster*[8]. Podophyllotoxin is reported to compete with colchicine in its effect on MTs[14]. Therefore, the glands have been exposed to podophyllotoxin prior to colchicine treatment (see Materials and Methods) to see if colchicine effect on 93D is modified. The data presented in Fig. 8b show that podophyllotoxin pretreatment has no effect on the induction of 93D by subsequent colchicine treatment (Fig. 7c): the level of the 93D activity in control (no podophyllotoxin treatment before colchicine) and podophyllotoxin followed by colchicine treated glands is similar; the chromosomal RNA synthesis is slightly more inhibited in latter glands (Fig. 8b).

Effect on MT poisons on cytoplasm—Preparations of MT poison treated glands show unusually abundant particles of various sizes in the cytoplasm (Fig. 9). These particles are most marked after chloral hydrate

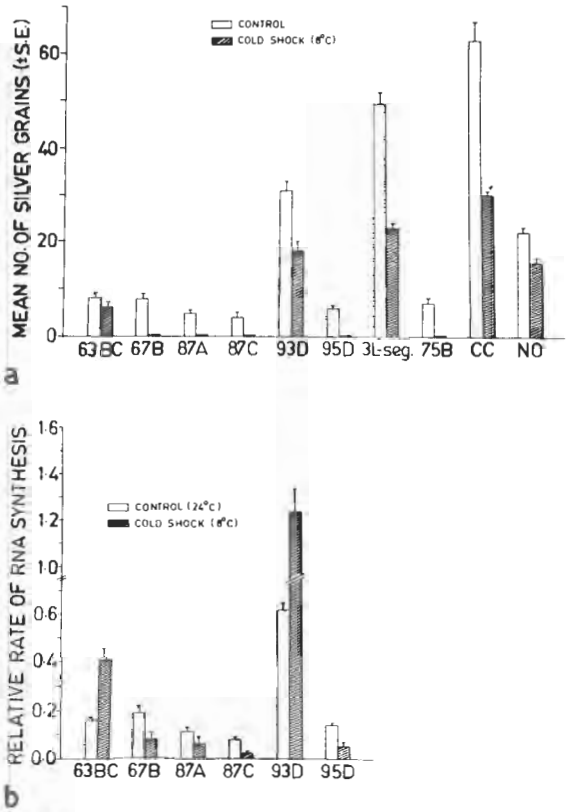

Fig. 4—Effect of cold shock on transcriptional activity in salivary gland polytene nuclei of *D. melanogaster* (a): ^{3}H-uridine incorporation (Mean no. of silver grains ±SE) on major HS puff sites, a developmentally active puff (75B), a chromosome segment of 3L (from 60A to 63A), chromocentre (CC) and on nucleolus (NO); (b): relative rate of ^{3}H-uridine incorporation on major HS puff sites in control or cold-shocked polytene nuclei. Relative rate = $\frac{\text{No. of silver grains on a HS puff}}{\text{No. of silver grains on the 3L segment (60A-63A)}}$. For each data point 35-35 nuclei were observed. For nucleolar grain density, grains were scored on three separate regions (each 25 μm^2) of nucleolus in a nucleus

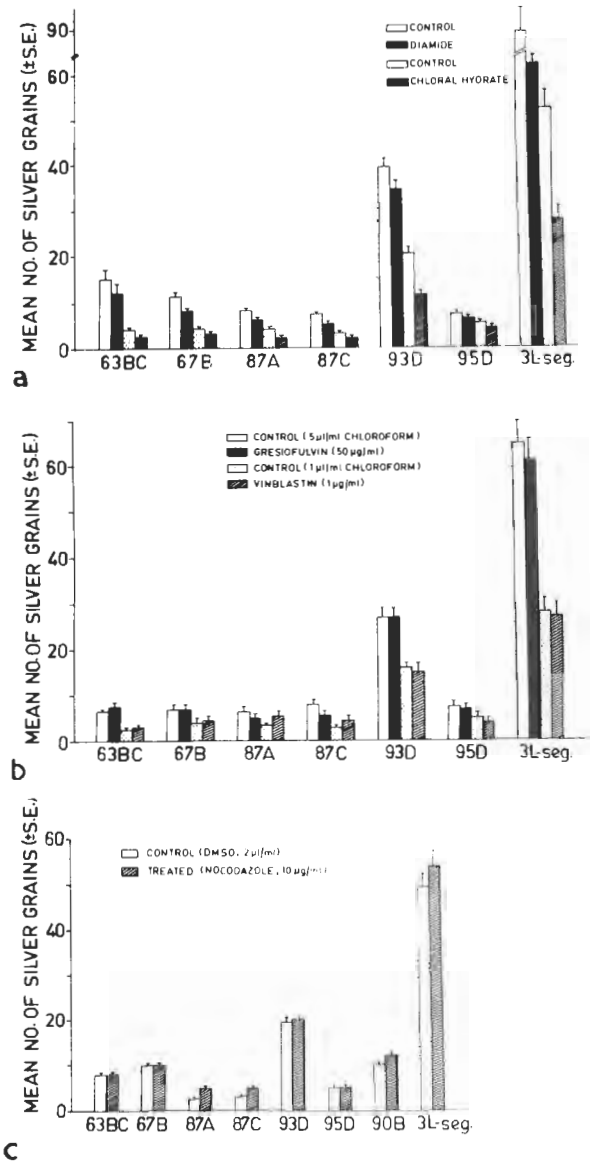

Fig. 6—Histogram showing effects of different microtubule poisons on ^{3}H-uridine incorporation on different HS puff sites, a developmentally active puff (90B) and on a segment of 3L (from 60A to 63A). Diamide and chloral hydrate treatment (a), griseofulvin and vinblastin treatment (b) and nocodazole treatment (c). In each case sister salivary glands incubated in medium having the solvent (when other than aqueous), as indicated, served as control

and diamide treatment (higher concentration) but are present with varying abundance in all glands treated with any of the MT poisons. The size and morphology of these particles are species specific but phylogenetically closer species appear to have more similar types thus the particles induced in cytoplasm of treated glands of *D. nasuta* and *D. hydei* are more similar to each other than to those seen in *D. melanogaster* and *D. ananassae* (Fig. 9).

Discussion

Earlier observation[8] that colchicine treatment causes a specific induction of the 93D puff in salivary glands of *D. melanogaster*, has stimulated the present study with different agents that disrupt the microtubular organization in cell so that the possible role of microtubles in modulating 93D puff activity could be understood.

In the present study, we have used a physical agent (cold shock) and several chemical ones in view of their known effects on depolymerization of MTs in different cell types[10]. Although the detailed mechanisms of action of all these MT poisons are not known, several of them are known to interact with tubulins or with the microtubule associated proteins (MAPs) in different ways and thereby prevent polymerization of MTs[10]. Colchicine binds to specific sites on tubulin dimers and thus blocks their polymerization in the dynamic

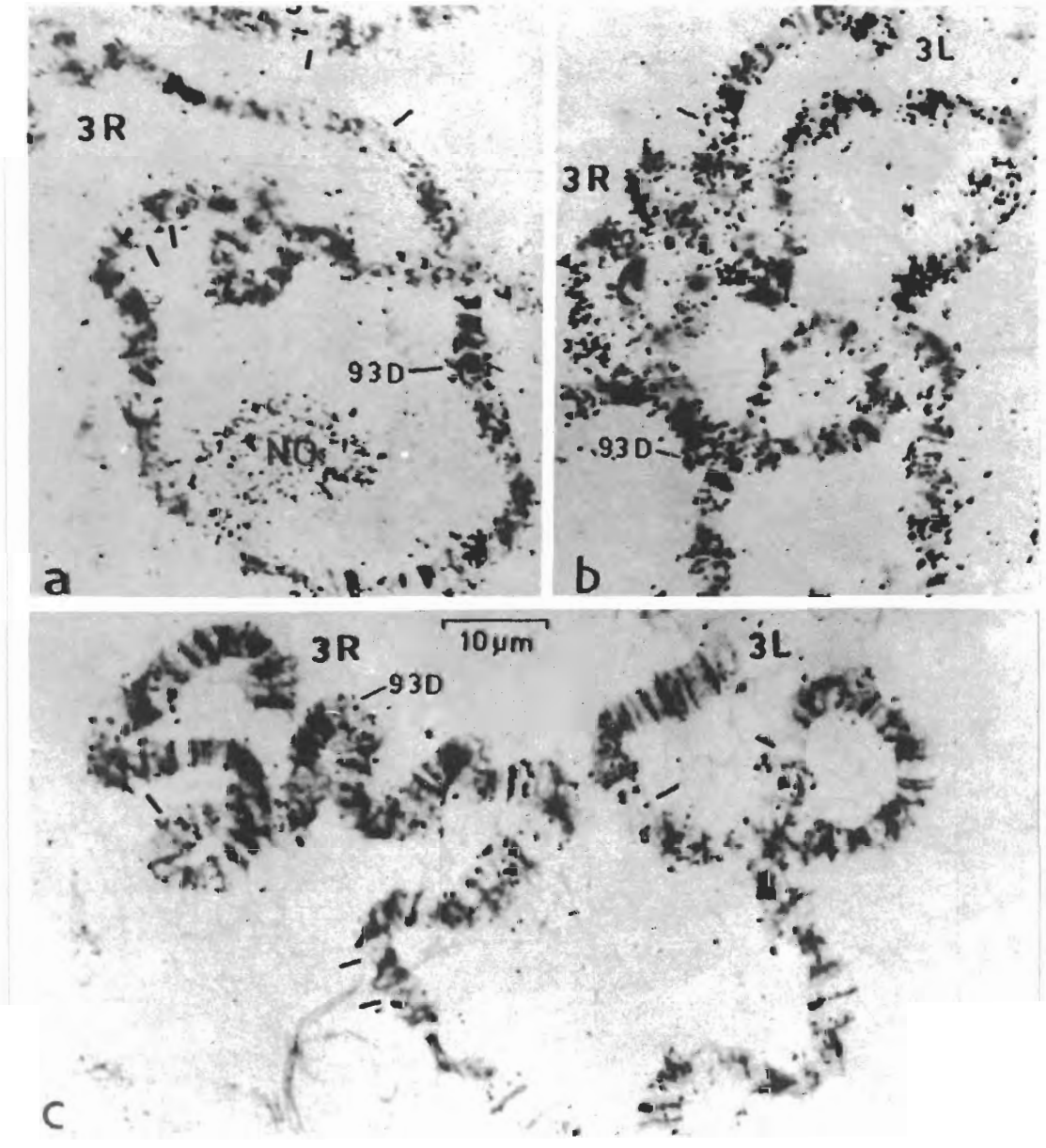

Fig. 5– ^{3}H-uridine labelling of polytene chromosomes of *D. melanogaster* in control (a), nocodazole (b) or chloral hydrate treated (c) salivary glands. HS puff sites other than 93D are indicated by—; NO = nucleolus

renewal process[10,14-16]. Podophyllotoxin's cytological effect as a spindle poison are similar to those of colchicine[14], and since podophyllotoxin inhibits colchicine binding to tubulin[14], it is presumed to act on MTs in a manner similar to that of colchicine[14]. Nocodazole's action upon MTs is also believed to be similar to colchicine action[17]. On the other hand, vinblastin and related derivatives interact in a different manner with tubulin protofilament and MAPs to affect normal polymerization of MTs[10].

Present results show that except the cold shock, none of the other MT poisons used in this study induce the 93D or any other heat shock puffs. The possibility that polytene cells are impermeable to MT poisons as used in this study is unlikely, since in all cases, the cytoplasm of treated cells gets filled with chracteristic particles (Fig. 9). Although the nature of these particles is not clear, they are presumably related to the common action of these chemicals on MTs: these particles may perhaps reflect some kind of crystallization of MTs analogous to effects known in other cell types[18-21]. An alternative explanation for the observed lack of induction of 93D in the MT poison treated glands may be that the chemicals were at a sub-threshold level since colchicine induced 93D puff activity[8] becomes apparent only at higher (5 μg/ml or higher) than the normal concentration (1 μg/ml or lesser) used for metaphase arrest[10]. Although this possibility cannot be ruled out, the present observations make it unlikely since lowest concentration of any chemical tried in this study (see Table 1) is on the higher side of the optimal concentration typically effective in disrupting MTs in other cell types[10]. Moreover, some of the MT poisons like chloral hydrate, podophyllotoxin, nocodazole, etc., were also tested at much higher concentration

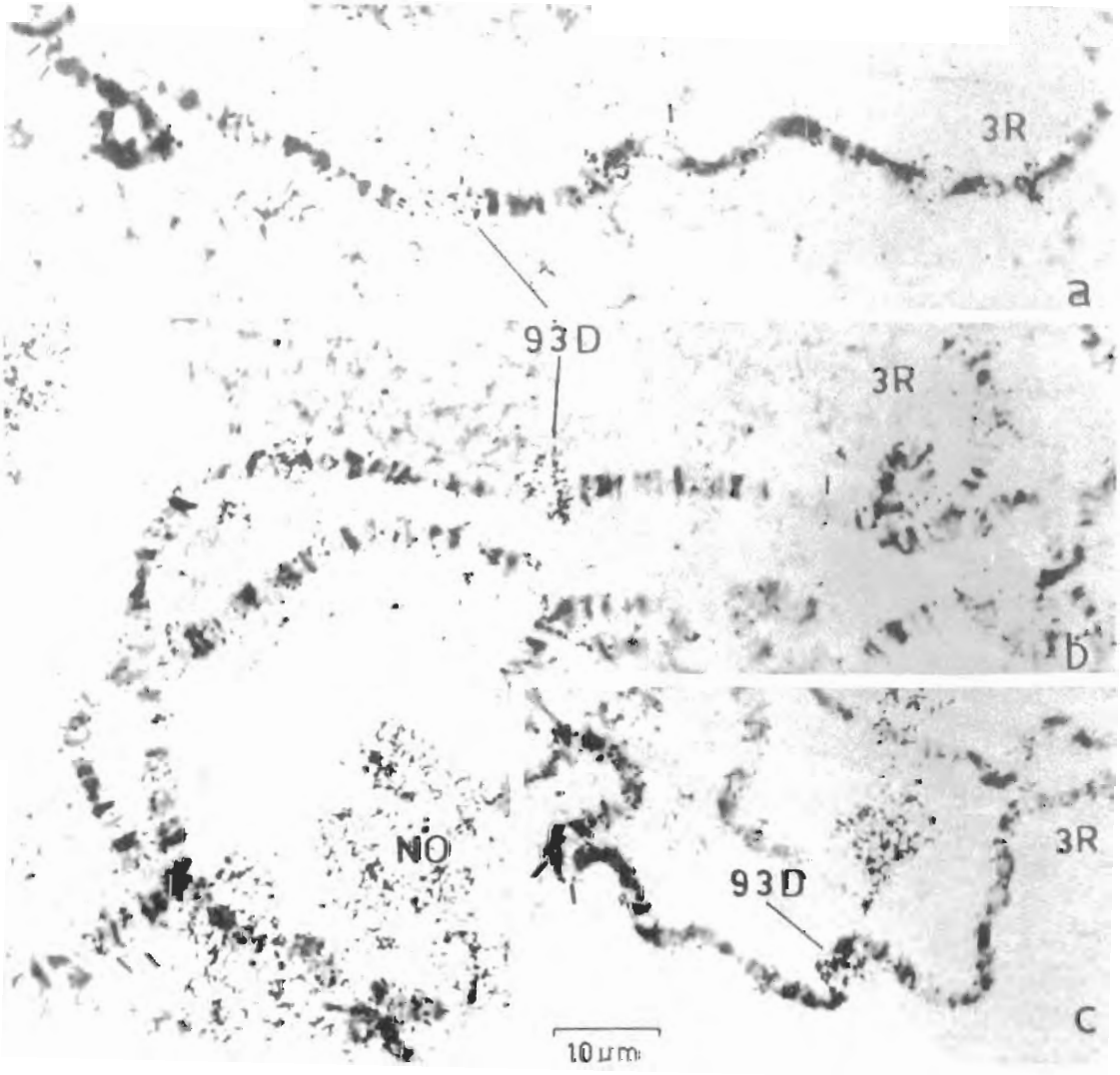

Fig. 7 Autoradiographs of chromosome arm 3R of *D. melanogaster* showing effect of podophyllotoxin (a), colchicine (b) or podophyllotoxin followed by colchicine treatment (c)

(Table 1), but in all cases the 93D puff of *D. melanogaster* or 93D-like puff in other species failed to respond. In view of these it is presumed that the 93D heat shock locus in *D. melanogaster* or 93D-like locus in other species of *Dorosophila* is insensitive to MT disruption, presumably caused by the various MT poisons used in this study. The present results also show that podophyllotoxin fails to inhibit colchicine action in inducing 93D when the two MT poisons are given together. If the 93D induction following colchicine treatment were due to the alkaloid's binding to MTs[10], podophyllotoxin could be expected to inhibit 93D induction by colchicine since podophyllotoxin competes for colchicine binding sites on MTs[14]. These results thus suggest that the MTs as such are not directly involved in modulating the 93D puff activity although the role of the other tubulin-like colchicine binding proteins remains distinctly possible as suggested by Lakhotia and Mukherjee[8].

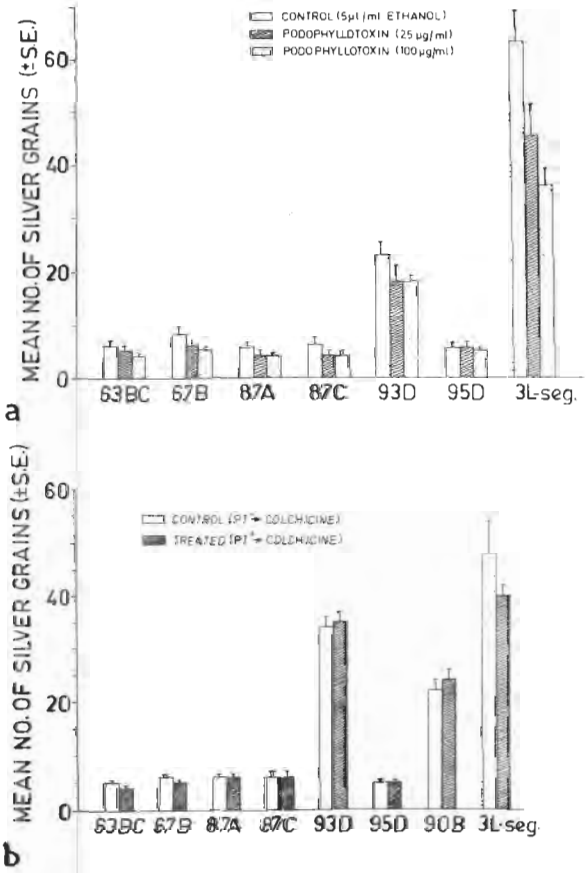

Fig. 8—^{3}H-uridine incorporation on major HS puff sites, a developmentally active puff (90B) and on a 3L chromosome segment (from 60A to 63A) after treatment with podophyllotoxin (a) or podophyllotoxin (PT) followed by colchicine (b)

In view of the above, it appears unlikely that the cold shock induced 93D-activity is due to depolymerization of MTs at low temperature. Instead, this is more likely to be a direct consequence of change in temperature itself since a "warm shock" (24°C) to salivary glands from 10°C-reared larvae also specifically activates the 93D locus (unpublished observation). Thus the 93D locus may act as a temperature monitor in the cell. However, it is not known whether its increased activity as a result of change in cellular temperature is its direct response or is modulated via other components of the cell. In any case, the inducibility of 93D by cold shock adds one more distinctive feature to this unique HS locus.

Petri *et al.*[22] reported that warming (22°C) of chilled (approximately 4°C) ovarian follicle cells of *D. melanogaster* causes induction of heat shock mRNAs as evidenced by their hybridization to major HS loci at 87A, 87C and 93D. However, in the present study, incubation of salivary glands at low temperature (4°C or 8°C) for 2 hr or recovery from the cold treatment was not found to induce the HS puffs except the 93D

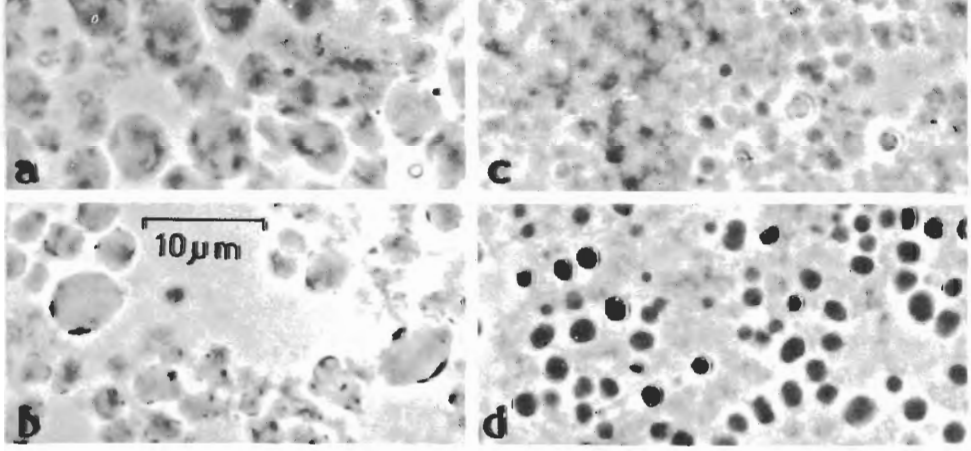

Fig. 9—Phase-contrast photomicrographs of cytoplasmic particles induced after chloral hydrate treatment in salivary glands of *D. melanogaster* (a), *D. ananassae* (b), *D. hydei* (c) and *D. nasuta* (d)

puff in the former case. In view of the present results and in view of a recent report of accumulation of a specific subset of *D. melanogaster* heat shock mRNAs during normal ovarian development without heat shock[23], it appears that the earlier observations of Petri *et al.*[22] were not related to induction of heat shock genes due to chilling or rewarming of the tissue.

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