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EARLY ACTION OF OESTROGEN ON THE INCORPORATION OF [^{3}H]URIDINE IN THE BLASTOCYST AND UTERUS OF RAT DURING DELAYED IMPLANTATION

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SUMMARY

The incorporation of [^{3}H]uridine into RNA in the blastocyst and uterus has been studied autoradiographically during delayed implantation and after treatment with oestrogen.

Minimal RNA synthesis, as judged by the accumulation of perchloric acid-precipitable isotope originating from [^{3}H]uridine, was seen in the blastocyst during delayed implantation. Treatment with oestrogen enhanced, within 5 min, incorporation of [^{3}H]uridine in a number of cells in all regions of the blastocyst; there was a fourfold increase in the number of grains/cell and the uptake was predominantly located in the nucleus. The number of grains/cell in the blastocyst gradually increased from fourfold at 15 and sixfold at 30 min to a maximal tenfold increase at 60 min after treatment with oestrogen.

Minimal RNA synthesis was seen in the uterus during delayed implantation. Treatment with oestrogen resulted, within 5 min, in a threefold increase in the number of labelled uterine epithelial cells and a sixfold increase in the number of grains/cell in the uterine epithelial nuclei; this level remained the same at 15, 30 and 60 min after hormone treatment. The significance of these results is discussed in relation to early action of oestrogen on the uterus and blastocyst. It is likely that activation of the delayed blastocyst may involve some mechanism which alters cell permeability.

INTRODUCTION

Earlier studies from this laboratory showed that oestrogen enhanced the synthesis of RNA, DNA and protein in the blastocyst and uterus of rats during delayed implantation (Mohla, 1968; Prasad, Dass & Mohla, 1968*a*; Prasad, Mohla & Rajalakshmi, 1968*b*; Dass, Mohla & Prasad, 1969). Treatment with oestrogen enhanced within 1 h the incorporation of [^{3}H]uridine into nuclear and cytoplasmic RNA in the blastocyst (Dass *et al.* 1969). In the uterus a significant increase in synthesis of RNA (as shown by incorporation of [^{3}H]uridine into the stromal cells) was observed 3 h after treatment with oestrogen and reached a peak at 6 h; it was maintained up to 12 h and declined at 18 h (Dass *et al.* 1969). The present experiments were therefore

designed to study autoradiographically the early action of oestrogen during the first 60 min period on the incorporation of [^{3}H]uridine into RNA in the blastocyst and uterus in the rat during delayed implantation.

MATERIALS AND METHODS

Colony bred, adult, virgin, female albino rats of the Holtzman strain (240–280 g) were maintained in light-controlled (14 h light and 10 h darkness) air-conditioned rooms (25 ± 1 °C) and fed a standard diet. Animals in pro-oestrus were caged with males of proved fertility and left overnight. Mating was confirmed by the presence of sperm in the vaginal smear the following morning which was considered as day 1 of pregnancy. Delayed implantation was caused by the method of Cochrane & Meyer (1957). Mated females were bilaterally ovariectomized on day 3 of pregnancy. On the day of ovariectomy and thereafter each rat received subcutaneously 4 mg progesterone (pregn-4-ene-3,20-dione)/day in 0·25 ml olive oil until the termination of the experiment. Surgical procedures were done under light ether anaesthesia in semisterile conditions.

Rats in which implantation was delayed experimentally by ovariectomy and progesterone treatment were used on day 9 after fertilization. They were divided into two groups, a control and an experimental group. The control denotes in all cases the condition during delayed implantation, the blastocysts are referred to as 'delayed blastocysts'. 'Experimental' refers to the condition after the administration of 17β-oestradiol (estra-1,3,5,(10)-triene-3,17β-diol) and the blastocysts are referred to as 'activated blastocysts'.

Administration of the precursor. The uteri were exteriorized by mid-ventral incision. The precursor ([^{3}H]uridine, sp.act. 6·0 Ci/mmol, Schwartz Bio Research; 1 μCi/uterine horn in 0·05 ml sterile 0·9 % NaCl solution) was instilled into the uterine lumen by means of a 26-gauge needle from the cervical end which was ligated after the withdrawal of the needle to avoid leakage into the peritoneal cavity.

Control group. The progesterone-treated controls received the precursor on day 9 of delayed implantation and were killed 15 min later.

Experimental group. Rats were injected subcutaneously with 1 μg 17β-oestradiol/rat in 0·08 ml olive oil on day 9 of delayed implantation and were killed, 15, 30 or 60 min after the injection of the hormone. All animals received [^{3}H]uridine 15 min before autopsy.

In one group of rats [^{3}H]uridine was instilled into the uterine lumen 15 min before autopsy as in other groups but 1 μg of 17β-oestradiol was injected subcutaneously 5 min before autopsy.

The animals were killed at the appropriate time and the uteri dissected free of fat and connective tissue. The uterine horns were flushed from the cervical ends with saline. The flushed blastocysts were washed quickly 2 or 3 times with saline, transferred to subbed slides, air-dried and fixed in Carnoy's fixative for 5 min. Some uteri were further washed, fixed in Carnoy's fixative for 1 h, embedded in paraffin, and sectioned at 4 μm. The blastocysts as well as sections of the uteri were treated with 2 % perchloric acid at 4 °C for 20 min to remove free nucleotides and were washed repeatedly in water. Sections of uteri and blastocysts of the control and experimental groups were incubated with ribonuclease (1 mg/ml at 37 °C for 2 h; ribonuclease,

salt-free lot no. 45846 FS was obtained from K & K Labs. New York) to confirm the specificity of incorporation of the precursor.

The slides were coated with diluted (1:2) Kodak nuclear track emulsion NTB[3] and the autoradiographs were developed after 7 and 21 days of exposure for blastocysts and uteri respectively. The autoradiographs were developed in Kodak D-19 developer for 5 min at 18 °C and acid-fixed. The slides were stained through the emulsion with buffered methyl green–pyronine or haematoxylin–eosin.

To count the number of uterine epithelial cells labelled, 500 cells/group from at least four animals were examined from sections of different parts of the uterus chosen at random and the cells showing the label were represented as the percentage of the total number of uterine epithelial cells counted.

The number of grains/cell was counted in 100 blastocyst or uterine epithelial cells. Any cell showing 3 to 4 grains above the background was scored as labelled.

RESULTS

The incorporation pattern of [^{3}H]uridine in the blastocyst and uterus after oestrogen treatment is shown in Table 1 and Plates 1 and 2.

Control group

During delayed implantation there was a minimal amount of RNA synthesis in the blastocyst (Pl. 1, fig. 1) and uterus (Pl. 2, fig. 5). In the delayed blastocyst the incorporation into nuclear RNA was seen in all regions of the blastocyst (average of 4 grains/cell). In the uterus the incorporation into nuclear RNA was seen in 29% of cells of the uterine epithelium with an average of 3 grains/cell.

Table 1. *Incorporation of [^{3}H]uridine by the blastocyst and uterus of virgin rats after 15 min of pulse labelling* (*means* ± S.E.M.)

		Blastocyst		Uterine epithelium	
Treatment	No. of rats	No.	Grains/cell	% cells labelled	Grains/cell
Control (delayed implantation)	16	45	4 ± 0·3	29 ± 7·2	3 ± 0·8
Oestrogen-treated, 5 min	6	13	18 ± 1·0	93 ± 0·4	20 ± 2·4
Oestrogen-treated, 15 min	5	14	18 ± 1·6	91 ± 1·5	19 ± 1·6
Oestrogen-treated, 30 min	9	24	24 ± 1·1	92 ± 1·6	20 ± 2·0
Oestrogen-treated, 60 min	5	14	42 ± 0·5	93 ± 2·0	15 ± 1·4

Experimental, oestrogen-treated groups

Treatment with oestrogen enhanced within 5 min the incorporation of [^{3}H]uridine into nuclear RNA in all regions of the blastocyst with an average of 18 grains/cell. The number of grains/cell increased to 24 and 42, 30 and 60 min respectively after administration of oestrogen; the grains were localized predominantly in the nuclei (Table 1 and Pl. 1, figs 2–4).

In the uterine epithelium the percentage of cells labelled and the number of grains/cell increased from 29 and 3 in the controls to 93 and 20 respectively within 5 min after treatment with oestrogen; the grains were predominantly in the nuclei. This pattern remained the same at 15, 30 and 60 min after oestrogen (Table 1; Pl. 2, figs 6 and 7).

DISCUSSION

Our results show that RNA synthesis in the uterus, as demonstrated by the uptake and incorporation of [^{3}H]uridine, reached an optimum level within 5 min after treatment with oestrogen; the grains were predominantly nuclear, suggestive of synthesis of nuclear RNA. These results provide autoradiographic support for the observations of Means & Hamilton (1966) who reported stimulation of synthesis of nuclear RNA and uptake of RNA precursors by the uterus within 2 min after the administration of oestrogen to ovariectomized adult rats. In their biochemical study, localization of the types of cells in which RNA synthesis was activated was not possible. However, our results show that among all the cells of the uterus, the cells of the uterine epithelium are most sensitive to the action of oestrogen.

In our earlier studies (Dass *et al.* 1969) on the incorporation of [^{3}H]uridine, it was seen that the stromal cells showed an increased uptake from 3 h after oestrogen treatment and reached a peak by 6 h which was maintained up to 12 h and declined by 18 h. The criterion used to evaluate the incorporation in the uterus was the labelling in the stromal cells since in both the uteri of the controls and the oestrogen-treated rats more than 90 % of the epithelial cells were heavily labelled. However, in the present experiments the dose of uridine (1 μCi/horn) was $\frac{1}{5}$ of the dose of the precursor used in our earlier studies. Using the percentage of labelled cells and the number of grains/cell as criteria for labelling it was possible to discern the pattern of incorporation in the cells of the uterus during the first 60 min after treatment with oestrogen. Our studies on the early time sequence of action of oestrogen on stimulation of RNA synthesis in the uterus show that the uterine epithelium is very sensitive to the action of oestrogen: RNA synthesis is enhanced in these cells within 5 min of the administration of the hormone and is followed later by a similar activation of the synthesis of RNA in the stromal cells.

In our earlier study (Dass *et al.* 1969) it was reported that incorporation of [^{3}H]-uridine into the cells of the delayed blastocyst was enhanced optimally 60 min after administration of oestrogen and remained at this level 3, 6, 12 and 18 h after treatment with the hormone. These studies highlight the differential time sequence of action of oestrogen on the uterus and blastocysts. It was suggested that the activation of the delayed blastocysts may be due to a direct action of oestrogen. An interesting question which arises from these studies concerns the mechanism by which a diapausing blastocyst is activated. During delayed implantation the blastocysts are viable and their oxygen consumption is not different from non-delayed ones (Gulyas & Daniel, 1967). RNA and protein synthesis are minimal (Prasad *et al.* 1968*b*) but very little synthesis of DNA occurs during this condition (Baevsky, 1964; Prasad *et al.* 1968*a*, *b*). Oestrogen reverses this dormancy and enhances the synthetic activities in the blastocysts (Mohla, 1968; Prasad *et al.* 1968*a*, *b*; Dass *et al.* 1969). Gwatkin (1966) observed that activation of delayed blastocysts (based on morphologically observable cellular growth) is facilitated by maintaining them in a medium containing basic amino acids. Psychoyos & Casimiri (1969) showed that the delayed blastocysts could be activated in terms of increased 'uptake' of [^{3}H]uridine by prolonged washing in a culture medium in the absence of oestrogen; they attribute this to removal of some inhibitory factor, which is presumably secreted by the uterus of a

progesterone-treated rat; however, their experiments do not specifically indicate the incorporation of [^{3}H]uridine into the acid-insoluble fraction (RNA). The results reported in the present study show that activation of the blastocyst as shown by RNA synthesis is optimally stimulated in the delayed blastocyst within 5 min of administration of oestrogen.

One of the earliest effects of oestrogen on the uterus is the release of cyclic AMP (adenosine 3′,5′-monophosphate) (Szego & Davis, 1967); they, Szego & Davis, showed that the increase in uterine cyclic AMP occurred within 15 s of intravenous administration of oestrogen. Griffin & Szego (1968) showed further that cyclic AMP *in vitro*, like oestrogen *in vivo*, also enhanced the incorporation of labelled amino acids, and their incorporation into proteins in segments of uterus of rat and it was postulated that cyclic AMP released as a result of treatment with oestrogen participates in the very early manifestation of the action of oestrogen on the uterus. Hechter, Yoshinaga, Halkerston & Birchall (1967) showed that cyclic AMP and other related nucleotides increase *in vitro*, uterine RNA and overall protein synthesis which is inhibited but not abolished by actinomycin D. They suggested that these nucleotides may be participating in the action of oestradiol on the uterus by activating metabolic processes operative at the gene and other loci in the cell. On the other hand, Sharma & Talwar (1970) observed that, *in vitro*, the effect of cyclic AMP is rapid and specific in facilitating uptake of precursors for RNA and protein synthesis by the uterus of rat. They concluded that the action of oestradiol on the uterus may involve steps which alter membrane structure and function by binding of the hormone to its receptor or are mediated by release of cyclic AMP or other biogenic amines. Cyclic AMP has been shown to alter membrane permeability (Orloff & Handler, 1962, 1967; Grantham & Burg, 1966).

Our results (Mohla & Prasad, 1970) show that cyclic AMP mimics the early action of oestrogen and enhances, within 15 min, the incorporation of [^{3}H]uridine into RNA in all the regions of the blastocyst; this uptake is localized predominantly in the nuclei. The uterine epithelial nuclei also show enhanced uptake of the RNA precursors at this time.

It is likely that the activation of the delayed blastocyst by oestrogen may involve, among other processes, some mechanism which alters cell permeability thus allowing increased availability of substrates for RNA synthesis.

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REFERENCES

Baevsky, U. B. (1964). The effect of embryonic diapause on the nuclei and mitotic activity of mink and rat blastocysts. In *Delayed implantation*, pp. 141–154. Ed. A. C. Enders. University of Chicago Press.

Cochrane, R. L. & Meyer, R. K. (1957). Delayed nidation in the rat induced by progesterone. *Proc. Soc. exp. Biol. Med.* **96**, 155–159.

Dass, C. M. S., Mohla, S. & Prasad, M. R. N. (1969). Time sequence of action of estrogen on nucleic acid and protein synthesis in the uterus and blastocyst during delayed implantation in the rat. *Endocrinology* **85**, 528–536.

Grantham, J. J. & Burg, M. B. (1966). Effect of vasopressin and cyclic AMP on permeability of isolated collecting tubules. *Am. J. Physiol.* **211**, 255–259.

Griffin, D. M. & Szego, C. M. (1968). Adenosine 3′5′ monophosphate stimulation of uterine amino acid uptake *in vitro*. *Life Sci.* **7**, 1017–1023.

Gulyas, B. J. & Daniel, J. C. (1967). Oxygen consumption in diapausing blastocysts. *J. cell. comp. Physiol.* **70**, 33–36.

Gwatkin, R. B. L. (1966). Amino acid requirements for attachment and outgrowth of the mouse blastocyst *in vitro*. *J. cell. comp. Physiol.* **68**, 335–343.

Hechter, O., Yoshinaga, K, Halkerston, D. K. & Birchall, K. (1967). Estrogen-like anabolic effects of cyclic 3′,5′ adenosine monophosphate and other nucleotides in isolated rat uterus. *Archs Biochem. Biophys.* **122**, 449–465.

Means, A. R. & Hamilton, T. H. (1966). Early estrogen action: concomitant stimulation within 2 minutes of nuclear RNA synthesis and uptake of RNA precursors by the uterus. *Proc. natn. Acad. Sci. U.S.A.* **56**, 1594–1598.

Mohla, S. (1968). Hormonal environment and blastocyst development in the albino rat. Ph.D. Thesis, University of Delhi.

Mohla, S. & Prasad, M. R. N. (1970). Stimulation of RNA synthesis in the blastocyst and uterus of rat by adenosine 3′5′-monophosphate (cyclic AMP). *J. Reprod. Fert.* (In Press).

Orloff, J. & Handler, J. S. (1962). The similarity of effects of vasopressin, adenosine-3′5′-phosphate (cyclic AMP) and theophylline on the toad bladder. *J. clin. Invest.* **41**, 702–705.

Orloff, J. & Handler, J. S. (1967). The role of adenosine 3′5′-phosphate in the action of antidiuretic hormone. *Am. J. Med.* **42**, 757–761.

Prasad, M. R. N., Dass, C. M. S. & Mohla, S. (1968*a*). The action of oestrogen on the blastocyst and uterus in delayed implantation—an autoradiographic study. *J. Reprod. Fert.* **16**, 97–104.

Prasad, M. R. N., Mohla, S. & Rajalakshmi, M. (1968*b*). Hormonal environment and blastocyst development. In *Proc. IIIrd Int. Congress of Endocr., Mexico*, pp. 939–944. *International Congress Series* No. 184. Amsterdam: Excerpta Medica Foundation.

Psychoyos, A. & Casimiri, V. B. (1969). Captation *in vitro* d'un precurseur d'acide ribonucleique (ARN) (uridine 5-^{3}H) par le blastocyste du rat; differences entre blastocystes normaux et blastocystes en diapause. *C. r. hebd. Séanc. Acad. Sci., Paris* **268**, 188–190.

Sharma, S. K. & Talwar, G. P. (1970). Action of cyclic AMP *in vitro* on the uptake and incorporation of uridine into RNA in the ovariectomised rat uterus. *J. biol. Chem.* **245**, 1513–1519.

Szego, C. M. & Davis, J. S. (1967). Adenosine 3′5′-monophosphate in the rat uterus. Acute elevation by estrogen. *Proc. natn. Acad. Sci. U.S.A.* **58**, 1711–1718.

DESCRIPTION OF PLATES

PLATE 1

Fig. 1. [^{3}H]Uridine incorporation in a few cells of a 'delayed' (control) blastocyst, showing a minimal number of grains in the nuclei. Methyl green–pyronine. (× 950.)

Fig. 2. [^{3}H]Uridine incorporation in a few cells of an 'activated' blastocyst 5 min after oestrogen treatment. A large number of grains are seen over the nuclei. Methyl green–pyronine. (× 950.)

Fig. 3. [^{3}H]Uridine incorporation in a few cells of an 'activated' blastocyst 60 min after oestrogen treatment. Heavy uptake is seen predominantly in the nuclei. Methyl green–pyronine. (× 950.)

Fig. 4. [^{3}H]Uridine incorporation in the 'activated' blastocyst 30 min after the administration of oestrogen. Uptake is seen in all regions of the blastocyst. Methyl green–pyronine. (× 650.)

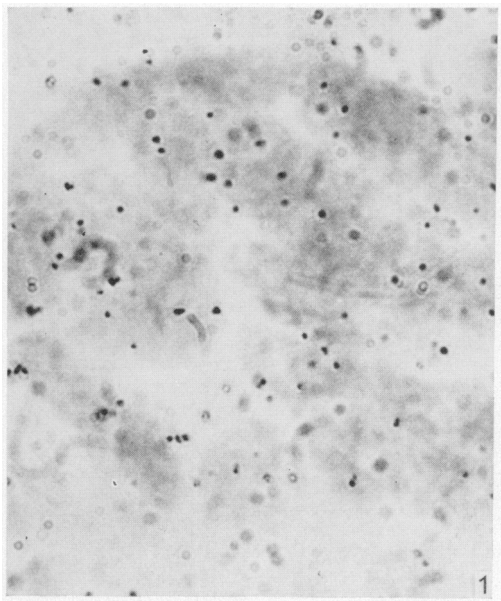
PLATE 2

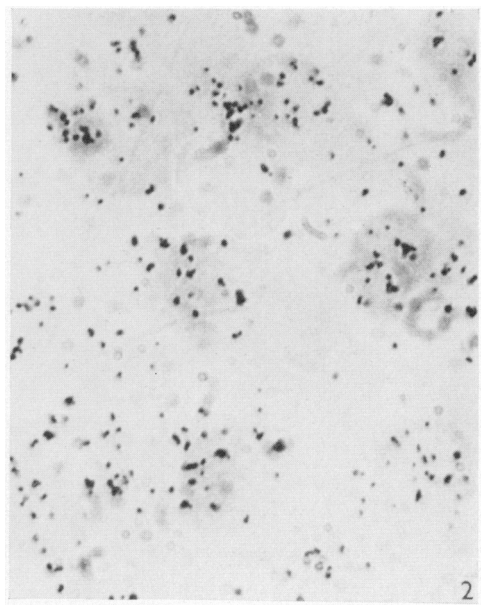
Fig. 5. [^{3}H]Uridine incorporation in uterine epithelium of a 'delayed' (control) rat. A few nuclei are labelled specifically. Haematoxylin and eosin. (× 1300.)

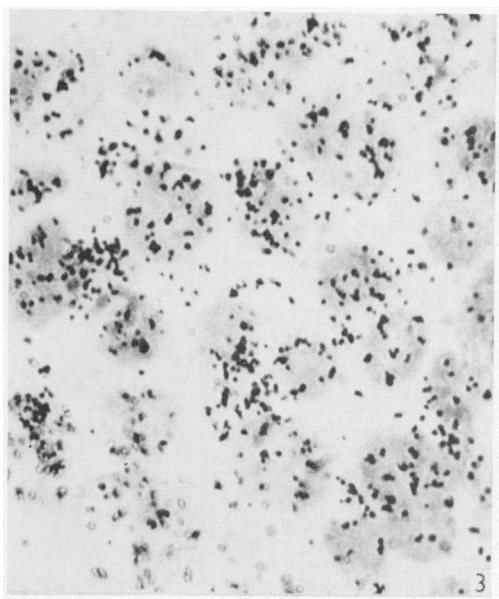
Fig. 6. [^{3}H]Uridine incorporation in the uterine epithelium 5 min after oestrogen administration. Uterine epithelial nuclei are fairly heavily labelled. Haematoxylin and eosin. (× 1300.)

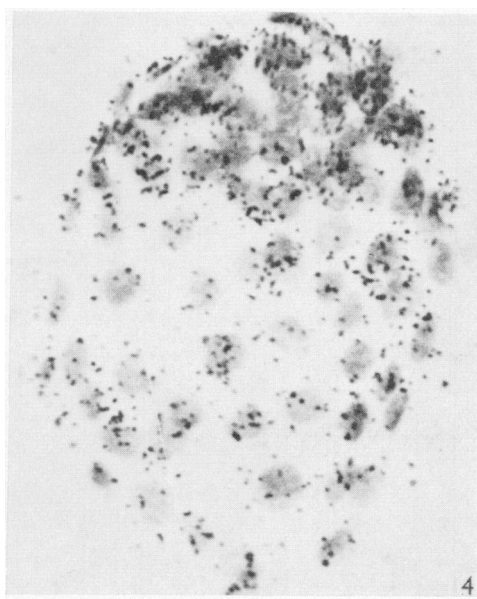
Fig. 7. [^{3}H]Uridine incorporation in the uterine epithelium 60 min after oestrogen administration. Heavy labelling is seen in epithelial nuclei. Haematoxylin and eosin (× 1300.)

Fig. 8. [^{3}H]Uridine incorporation in the blastocyst of a 'delayed' rat 60 min after administration of oestrogen. The uptake is heavy in all regions of the blastocyst. Methyl green–pyronine. (× 650.)









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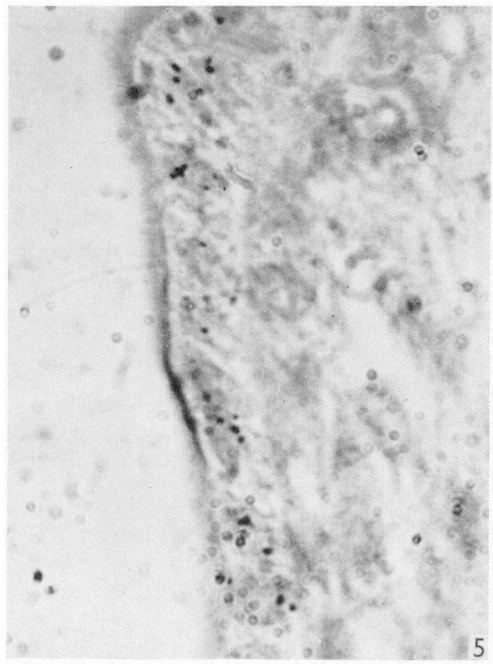
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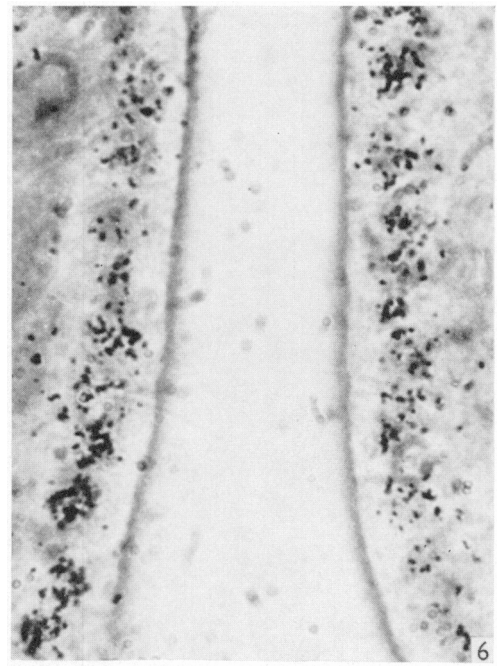
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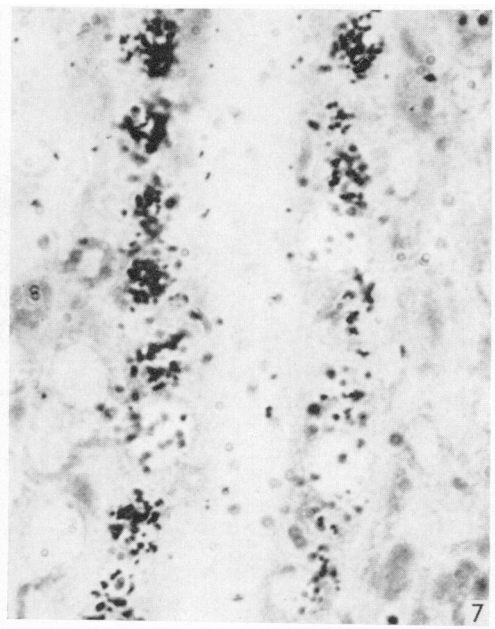
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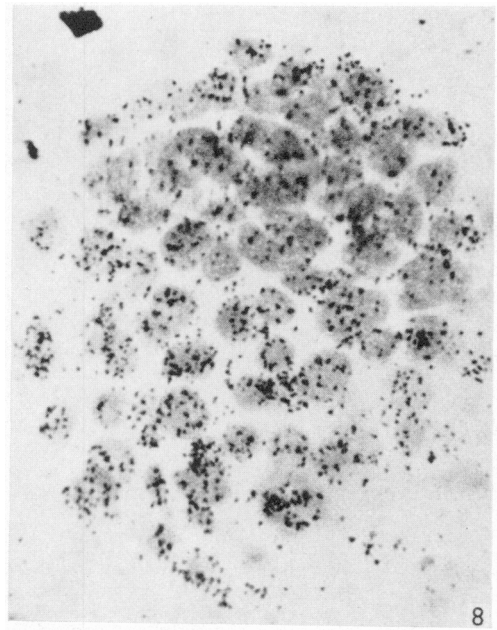
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