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Effects of the nucleic acid synthesis inhibitors on the cell division and chromosome structure of *Vicia faba* L.

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Two'-deoxyadenosine (2'-AdR), 5-fluorodeoxyuridine (FUdR), formycin, and 3'-deoxyadenosine (3'-AdR) are all analogues of nucleosides, and they inhibit the synthesis of nucleic acids. The effects of these four chemicals on the cell division and chromosome structure in *Vicia faba* root meristematic cells were investigated. It seemed that the cytological effects were different between the inhibitors of DNA synthesis, 2'-AdR and FUdR, and the inhibitors of RNA synthesis, formycin and 3'-AdR. The former two chemicals disturbed the progression of S phase cells owing to the inhibitory effects on DNA synthesis. Chromosome aberrations which mostly consisted of breaks or gaps were induced by the treatments of these two chemicals. Mitotic inhibitions were also obtained by the treatment with latter two chemicals, formycin and 3'-AdR, and they were not restricted in interphase, but occurred in prophase or metaphase. Structural changes in chromosomes were characterized by the localization of isochromatid breaks in secondary constrictions, and by the appearence of extreme contraction of chromosomes in both metaphase and anaphase. The difference of the mechanisms inducing chromosome aberrations between the inhibitors of DNA and those of RNA synthesis was discussed.

With the progress of biosynthetic researches on nucleic acids a number of inhibitors of nucleic acid synthesis have been found. Among them, 2'-deoxyadenosine (2'-AdR), 5-fluorodeoxyuridine (FUdR), formycin and 3'-deoxyadenosine (3'-AdR, cordycepin) are all analogues of nucleosides (Fig. 1).

Two'-AdR is phosphorylated in the cells to 2'-deoxyadenosine triphosphate (2'-dATP) and then this inhibits the deoxydization of ribonucleoside diphosphate to deoxyribonucleoside diphosphate, leading to the deficiency of the precursors of DNA, and results in the inhibition of DNA synthesis (REICHARD, 1968).

FUdR is also phosphorylated in the cells to 5-fluorodeoxyuridine monophosphate (F-dUMP) and this blocks the reaction of thymidylate synthetase, causing to the deficiency of thymidylate, the precursor of DNA, and con-

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sequently results in the inhibition of DNA synthesis (HARTMANN and HEIDELBERGER, 1961).

Formycin, an antibiotic isolated from culture filtrates of Actinomycetes, *Nocardia interforma* (HORI *et al.*, 1964), is phosphorylated to formycin triphosphate (Fo-TP). Fo-TP is incorporated to the cells and inhibits the synthesis of phosphoribosylpyrophosphate (PRPP). In the process of DNA transcription Fo-TP affects so as to reduce the rate of chain initiation and inhibits the release of growing RNA chains from DNA, and results in the inhibition of RNA synthesis (ROY-BURMAN, 1971, DARLIX *et al.*, 1971). YANAGISAWA *et al.* (1977) reported that formycin prohibits the syntheses of all RNA species including polyadenylic acid-containing RNA.

Three'-AdR, an antibiotic isolated from *Cordyceps*, is also phosphorylated to 3'-dATP and inhibits the synthesis of PRPP, and because of 2'-5' internucleotide linkage, 3'-dATP disturbs the growing of RNA chain during transcription. Thus 3'-AdR inhibits the synthesis of RNA. Besides, 3'-AdR inhibits DNA synthesis, through the inhibition of deoxydization of ADP and GDP and reaction of DNA polymerase (ROY-BURMAN, 1970).

It is known that the treatments of 2'-AdR (KIHLMAN, 1963), FUdR (TAYLER *et al.*, 1962), and 3'-AdR (KIHLMAN and ODMARK, 1966) produce chromosome aberrations in higher plant cells. The mechanisms of the induction of chromosome aberrations by these chemicals have not been clarified.

The present investigation deals with the examination of the effects of the above four chemicals on the cell division and chromosome structure of *Vicia faba* root cells and the comparison of characteristic effects of these chemicals. And the possible correlation between cytological and biochemical effects of these chemicals will be discussed.

Materials and Methods

The seeds of *Vicia faba* L. were soaked for 24 hr in tap water, and then were allowed to germinate in moist Vermiculite for 4 days at 20°C. The main roots which were 4-5 cm long were used in the experiments.

The concentrations of chemicals were $1.5\text{-}4\times10^{-3}$ M for 2'-deoxyadenosine, 10^{-7}-10^{-5} M for 5-fluorodeoxyuridine, 10^{-6}-10^{-3} M for formycin and 10^{-4}-5×10^{-4} M for 3'-deoxyadenosine. These chemicals were dissolved in deionized water immediately before use.

After ten seedlings were soaked for available time in these chemical solutions, they were transferred in aerated tap water. Root tips were excised, fixed in ethanol-acetic acid (3 : 1) at various recovery times, hydrolyzed in

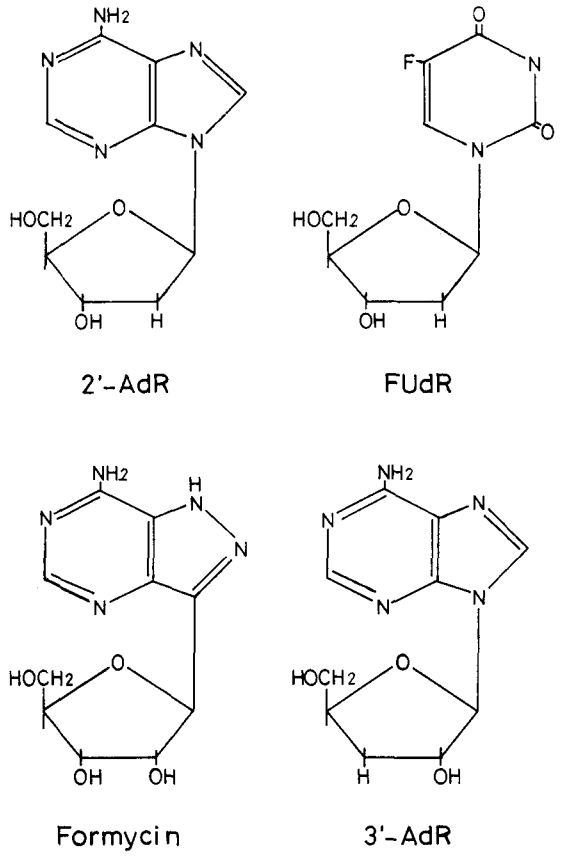

Fig. 1. Chemical structures of 2′-AdR, FUdR, formycin and 3′-AdR.

1 N HCl at 60°C for 8 min, and stained by Feulgen staining reaction. Squash preparations were made of the distal 1.5 mm portion of the roots. When chromosome aberrations were examined, root tips were pretreated with 0.05% colchicine for 2 hr prior to the fixation.

Observations were carried out mainly both about the presence or absence of mitotic delay and about the types and frequencies of chromosome aberrations. The progression of cell division in root meristems was estimated by a mitotic index; i. e., the ratio of number of mitotic cells to total number of observed cells.

To determine the division stage of cells treated with chemicals some roots were exposed to tritiated thymidine (5-10 μCi/ml) for 2 hr at the same time as the treatment. For autoradiography preparation slides were covered in Fuji stripping films, allowed to dry and stored for 2-4 weeks

in the dark at 4°C. They were developed in Kodak 19 b.

Results

1. *Effect of 2′-deoxyadenosine*

When the seedlings were treated with 4×10^{-3} M 2′-AdR for 2 hr, cell divisions were inhibited at 0～8.5 hr after the treatment. However, mitotic indices recovered normal level at about 10 hr after the treatment, subsequently keeping higher level bearing maximum peak at 14 hr (Fig. 2).

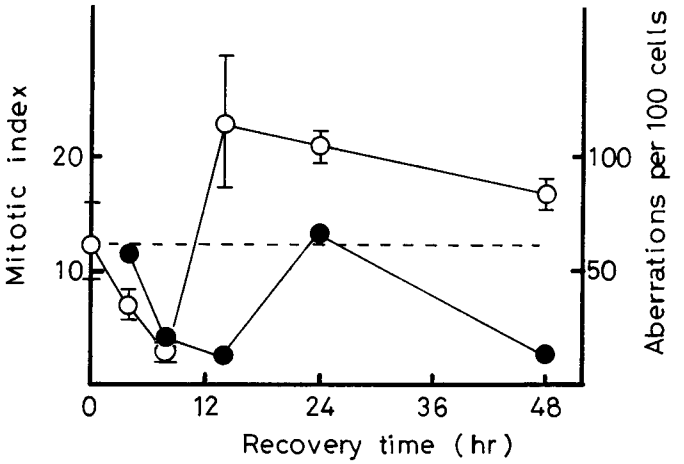

Fig. 2. Mitotic indices and frequencies of chromosome aberrations induced by the treatment with 4×10^{-3} M 2′-AdR for 2 hr at various recovery times. ○—○, mitotic indices of cells treated with 2′-AdR; ----, mitotic index of untreated control cells; ●—●, frequencies of chromosome aberrations induced by 2′-AdR.

Chromosome aberrations increased remarkably at 4 and 24 hr after the treatment. Table 1 shows frequencies and types of chromosome aberrations induced by the treatment with 4×10^{-3} M 2′-AdR for 2 hr. Most of these aberrations were break types (breaks and gaps), and an exchange type rarely existed. At 4 hr after the treatment, gaps were predominant aberrations (*i. e.*, gaps occupied 80% of total aberrations), while at 24 hr breaks were predominant (*i. e.*, breaks occupied 64% of thetotal) Furthermore isochromatid breaks were more frequent than chromatid breaks.

When root tips were fixed at 4 hr after the treatment with ^{3}H-TdR and 2′-AdR simultaneously, ^{3}H-TdR-labeled metaphase chromosomes were not observed (Plate I), indicating that the stage when the 4-hr-cells were treated with 2′-AdR was not at S-phase but probably at G_2 phase.

TABLE 1. Frequencies and types of chromosome aberrations induced by the treatment with 4×10^{-3} M 2′-AdR for 2 hr

Recovery time (hr)	Observed metaphase cells	Abnormal metaphase cells (%)	Aberrations per 100 cells					
			Total	B″	B′	G″	G′	Exchanges
4	101	40	63	5	7	19	31	1
8.5	103	16	18	1	3	2	12	0
14	104	5	14	11	2	0	1	0
24	103	28	58	27	10	7	14	0
48	112	12	17	5	1	4	5	2
Control	105	2	2	0	0	1	1	0

B″ and B′: Isochromatid and chromatid breaks, respectively.
G″ and G′: Isochromatid and chromatid gaps, respectively.

When seedlings were treated with 1.5×10^{-3} M 2′-AdR for 24 hr and then recovered in water for 25–32 hr, chromosome aberrations of exchange types, though only a few exchange was observed in secondary constrictions in M chromosomes.

In PIate I-c & I-d the aberrations of break types and exchange type induced by the treatments with 2′-AdR are shown.

2. *Effects of 5-fluorodeoxyuridine*

The seedlings were treated with 10^{-5}, 10^{-6} and 10^{-7} M FUdR for 2 hr respectively, and then recovered in tap water for up to 22 hr.

At concentrations of 10^{-5} and 10^{-6} M the effects on mitotic inhibition were so strong that mitoses were completely disturbed at 12–22 hr (Fig. 3). At a concentration of 10^{-7} M the inhibition was disappeared at 22 hr of the recovery time, although mitosis was inhibited during 0–12 hr of the recovery time.

As appeared in Table 2, the types of chromosome aberrations induced by FUdR were all the break types, *i. e.*, breaks and gaps, and the aberrations increased according with the increase in the concentrations of FUdR. Plate II shows the aberrations induced by the treatment with FUdR; Plate II-a shows isochromatid and chromatid breaks and Plate II-b shows a pulverization of chromosomes which were often observed at 12 hr after the treatment.

The autoradiographic observations indicated that the metaphase chromosomes at 4 hr of the recovery time were unlabeled, and then the division stage of concerned cells were G_2 phase when they were treated with FUdR.

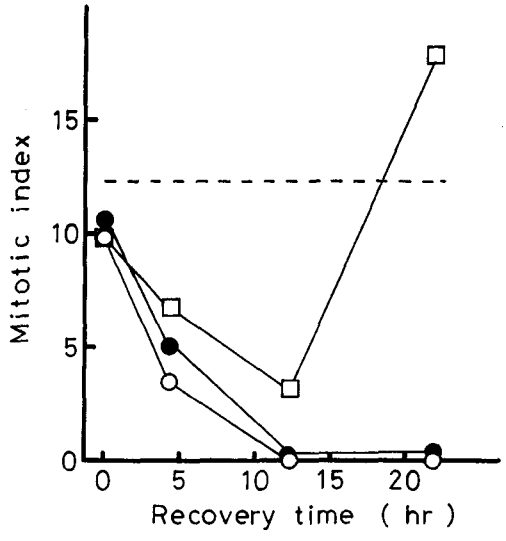

Fig. 3. Mitotic indices at various recovery times after the treatments with 10^{-7}, 10^{-6} and 10^{-5} M FUdR for 2 hr. □—□, 10^{-7}M; ●—●, 10^{-6} M; ○—○, 10^{-5} M; - - - -, control.

TABLE 2. Frequencies and types of chromosome aberrations induced by the treatments with FUdR at concentrations of 10^{-5}, 10^{-6} and 10^{-7} M for 2 hr

Concentration of FUdR (M)	Recovery time (hr)	Observed metaphase cells	Abnormal metaphase cells (%)	Aberrations per 100 cells				
				Total	B″	B′	G″	G′
10^{-5}	4	109	82	223	16	66	63	77
10^{-6}	4	89	47	103	11	28	36	28
10^{-7}	4	103	24	26	1	1	8	16
	12	105	27	30	0	3	5	22
	22	102	31	43	1	3	15	24
Control	—	210	1	1	0	0	0.5	0.5

B″ and B′: Isochromatid and chromatid breaks, respectively.
G″ and G′: Isochromatid and chromatid gaps, respectively.

Since FUdR is the inhibitor of the synthesis of TMP, the precursor of DNA, it may be expected that the effects of FUdR on cells might become more slight or disappear by the addition of thymidine (TdR), the precursor of TMP, at the same time to the treatment with FUdR. As might have been expected, when seedlings were treated simultaneously with 10^{-6} M

FUdR and 10^{-4} M TdR for 2 hr, and transferred in water for up to 22 hr, the mitosis was rather recovered, although the mitotic indices were lower than those of untreated seedlings (Fig. 4). The frequency of chro-

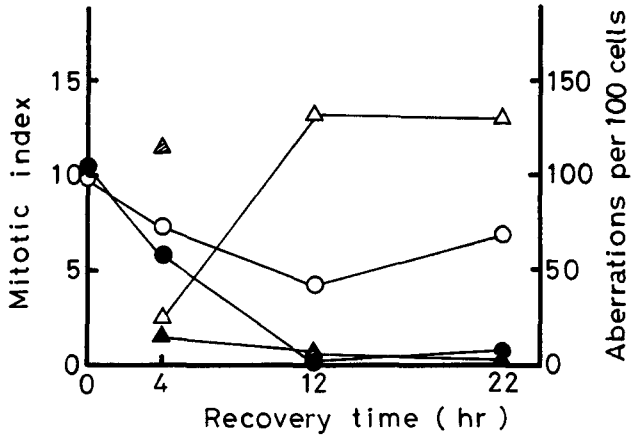

Fig. 4. Mitotic indices and frequencies of chromosome aberrations induced by the treatment with 10^{-6} M FUdR and 10^{-4} M TdR, simultaneously. ○—○, mitotic indices of cells treated with FUdR and TdR, simultaneously; ●—●, mitotic indices of cells treated with FUdR; △—△, the frequencies of chromosome aberrations induced by the simultaneous treatment with FUdR and TdR; ▲—▲, frequencies of chromosome aberrations induced by the treatment with TdR; ◬, the frequency of chromosome aberrations induced by the treatment with FUdR.

TABLE 3. Frequencies and types of chromosome aberrations induced by the treatment with 10^{-6} M FUdR alone or simultaneous treatement with 10^{-6} M FUdR and 10^{-4} M TdR

Treatments	Recovery time (hr)	Observed metaphase cells	Abnormal metaphase cells (%)	Aberrations per 100 cells				
				Total	B″	B′	G″	G′
FUdR	4	89	47	103	11	28	36	28
FUdR+TdR	4	67	19	22	0	5	1	16
TdR	4	103	17	17	0	0	6	11
FUdR+TdR	12	102	66	131	9	23	34	65
TdR	12	99	7	7	0	0	1	6
FUdR+TdR	22	108	75	132	6	15	38	73
TdR	22	71	3	2	0	0	1	1

B″ and B′: Isochromatid and chromatid breaks, respectively.
G″ and G′: Isochromatid and chromatid gaps, respectively.

mosome aberrations decreased drastically to one fifth of total aberrations induced by FUdR alone at 4 hr of the recovery time. At 12- and 22-hr of the recovery times, although could not be compared with the treatment with FUdR alone, many aberrations occurred, especially gaps were predominant instead of breaks (Table 3). These results suggest that the effects of 10^{-6} M FUdR on the cell division and on the induction of chromosome aberrations were considerably suppressed, at least at 4 hr after the treatment, by the addition of TdR whose concentration was 100 times greater than that of FUdR.

3. *Effects of formycin*

The seedlings were treated with formycin at concentrations in the range from 10^{-6} M to 5×10^{-4} M for 2 hr. As shown in Fig. 5, mitotic inhibitions occurred at the concentration above 5×10^{-6} M. The mitotic inhibition induced by 5×10^{-4} M formycin was disappeared at 48 hr after the treatment. The percentage of prophase cells to the total numbers of mitotic cells were considerably higher in treated cells, showing 60-80%, than 50% in untreated cells. These results may show that the progressions of cell division cycle are inhibited not only from interphase to mitotic phase, but also from prophase to metaphase.

As shown in Table 4, most of the chromosome aberrations induced by formycin belonged to break types, especially isochromatid breaks and both isochromatid and chromatid gaps. The remarkable result was that the most of isochromatid breaks (82% of the total breaks) were localized in the secondary constrictions of M-chromosomes. In spite of the treatment with

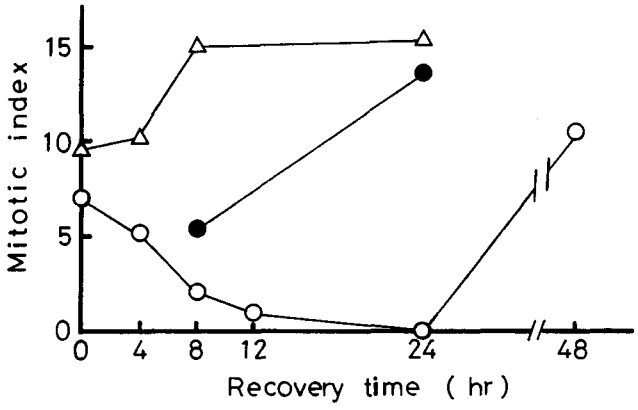

Fig. 5. Mitotic indices at various recovery times after the treatments with 10^{-6}, 5×10^{-6} and 5×10^{-4} M formycin for 2 hr. △—△, 10^{-6} M; ●—●, 5×10^{-6} M; ○—○, 5×10^{-4} M.

TABLE 4. Frequencies and types of chromosome aberrations induced by the treatment with formycin or 3′-AdR

Chemicals	Observed metaphase cells	Abnormal metaphase cells (%)	Aberrations per 100 cells						
			Total	B″	in NC (%)	B′	G″	G′	Exchange
Formycin	387	50	76	28	82	2	20	26	1
3′-AdR	278	44	85	61	99	12	5	7	0

B″ and B′: Isochromatid and chromatid breaks, respectively.
G″ and G′: Isochromatid and chromatid gaps, respectively.
NC: Nucleolar constriction.

higher concentrations of formycin breaks were very few in any other regions in M-chromosomes and in 10 S-chromosomes.

By the treatment with formycin the extreme contraction of both metaphase and anaphase chromosome was observed. As shown in Plate III, the degree of the contraction induced by formycin (5×10^{-4} M) was larger than that induced by colchicine (0.05%). The contraction of chromosomes occurred immediately and 4 hr after the onset of treatment, indicating that the contraction might occur in G_2 stage and prophase. These contractions, however, disappeared at 48 hr after the removal of formycin.

4. *Effects of 3′-deoxyadenosine*

Mitotic inhibition induced by the treatments with 3′-AdR was not so strong as was observed in formycin treatment; *i. e.*, even when treated with at a concentration of 5×10^{-4} M for 24 hr the mitotic index showed 5.3 ± 1.21. When treated for 2-6 hr an accumulation of prophase cells was observed, and when treated for 24 hr an accumulation of metaphase cells was observed; *i. e.*, frequencies of prophase cells of total mitotic cells were 70-80% in the former case, and those of metaphase cells in the latter case were 40%, while in the case of untreated cells prophase and metaphase cells occupied 50% and 10% of total mitotic cells respectively.

As similarly as observed in the case of formycin, the extreme contractions of both metaphase and anaphase chromosome were observed in the treatment with 3′-AdR (Plate III-c). And the binucleate cells were also observed (Plate III-e). In the contracted anaphase chromosomes, it was observed that the centromeric regions were stretched or broken (Plate III-d). As shown in Table 4, 99% of total isochromatid breaks were localized in the secondary constrictions. Gaps were so few as to occupy only 14% of total aberrations.

Discussion

The inhibitors of nucleic acid synthesis, 2′-AdR, FUdR, formycin and 3′-AdR used here, all inhibited the progression of mitosis of root meristematic cells in *Vicia faba*. These inhibiting effects, however, were reversible, *i. e.*, the mitosis recovered gradually with the lapse of time after the removal of these chemicals.

The inhibiting effects on the cell division were different between both 2′-AdR and FUdR, and both formycin and 3′-AdR. The former two chemicals, the inhibitors of DNA synthesis, disturb the proceeding of cells from interphase to mitotic phase. It has been interpreted that these inhibitory effects were the consequence of the inhibition of the progression of S phase, owing to the inhibiting effect of DNA synthesis, as reported by KIHLMAN and ODMARK (1966), and KIHLMAN and HARTLEY (1967).

The other two chemicals, formycin and 3′-AdR, inhibited the progressions of cell cycle not only from interphase to mitotic phase but also from prophase to metaphase or from metaphase to anaphase. The accumulation of prophase or metaphase cells may suggest that specific RNA or protein synthesis may be required for the progression of cells from prophase to metaphase or from metaphase to anaphase. The accumulation of prophase cells by 3′-AdR in *Allium cepa* was investigated by GONZÁLEZ-FERNÁNDEZ *et al.* (1970), and they reported that a synthesis of RNA, probably specific, required to enable the cells to go on from prophase to metaphase of mitosis, took place during prophase. These specific RNA or protein synthesis, however, have not yet been clarified.

The effects of these chemicals on the induction of chromosome aberrations and on the structural changes of chromosomes were also different between both 2′-AdR and FUdR and both formycin and 3′-AdR, and these results might be the consequence of the inhibiting effect on each nucleic acid synthesis.

When cells were treated with the former two chemicals, 2′-AdR and FUdR, breaks and gaps were predominant types of the chromosome aberrations. Frequencies of both gaps and breaks increased according with the increase in the concentration of FUdR (Table 2). By the addition of TdR simultaneously with FUdR, the frequency of chromosome aberration was drastically decreased at 4 hr after the treatment. It seemed that this decrease in chromosome aberrations is the consequence of the recovery of DNA synthesis. At 12- and 22-hr after the treatment, many aberrant chromosomes were observed (Fig. 4), and gaps were predominant aberrations.

Therefore it is likely that chromosome aberrations induced by 2′-AdR or FUdR may occur as a result of the lesions in DNA molecules, and the degree of lesions may cause to produce either gaps or breaks.

Since many aberrant chromosomes could be induced in G_2 phase, DNA synthesis, which was not only quantitatively but also qualitatively different from that in S phase, may occur in G_2 as reported by ODMARK and KIHLMAN (1965).

On the other hand chromosome aberrations induced by formycin and 3′-AdR were characterized by the localization of isochromatid breaks in the secondary constriction in M chromosome. Breaks were very few in any other regions than the secondary constrictions in M chromosomes and other chromosomes. As KIHLMAN and ODMARK (1966) have speculated that these localization of breaks may be due to the inhibitory effects of these chemicals on the ribosomal RNA synthesis occurring in the secondary constrictions where the cistrons for rRNA are contained. It seems that gaps may be the consequence of folding defect by the inhibition of RNA or protein synthesis. Another characteristic change of the chromosome structure was an extreme contraction. This change may be also induced by the inhibition of RNA or protein synthesis. Since the contraction seems to occur in G_2 phase, RNAs synthesized in G_2 may play a part in the regulation of contraction.

There have been a lot of investigation dealing with the nature of gaps, but they have been yet controversial.

SCHEID and TRAUT (1970, 1971 a, b, c, 1973) reported a series of investigations dealing with the nature of X-ray induced achromatic lesions ("gaps") in *Vicia faba*. They studied it by UV-microscopy, scanning electron microscopy, staining of chromosomal proteins, DNA and/or RNA, and measuring the chromatid length. And they argued that gaps reflected a loss of chromosomal material or a despiralization.

HITTELMAN and RAO (1974) observed two types of gaps using premature chromosome condensation (PCC) method in Chinese hamster ovary cells. It is suggested by them that one type was due to a structural discontinuity within a chromatid and the other was due to the failure of a proper degree of condensation caused by altered DNA-protein binding.

BRØGGER (1975), and BRØGGER and WAKSVIK (1978) reported that mercaptoethanol induced gaps in human lymphocyte chromosomes were the consequence of folding defects.

BRINKLEY and HITTELMAN (1975) argued from the electron microscopic observation on two types of "gap"; *i.e.*, "gap" shown as true structural

continuity and that shown as true structural discontinuity.

Our results also show that there may be two types of gaps which cannot be distinguished by the light microscopic observations. It is supposed that one type results from lesions occurred in DNA molecules and the other type results from folding defect.

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Plate I.

a and b Seedlings were treated with 4×10^{-3} M 2′-AdR and ^{3}H-TdR simultaneously for 2 hr, and root tips were fixed at 4 hr after the end of the treatment.

(a) A labeled interphase cell.

(b) An unlabeled metaphase cell.

c and d Seedlings were treated with 1.5×10^{-3} M 2′-AdR for 24 hr, and root tips were fixed at 24 hr after the end of the treatment.

(c) Two isochromatid gaps (→) and a chromatid gap (▶) all occurred in the middle part in S-chromosomes. An intra-arm exchange (➤) was observed in the distal part of a short arm in M-chromosome.

(d) An interchromatid exchange (➤) occurred between the distal part of the long arm in M-chromosome and the proximal part of the long arm in S-chromosome.

Plate II.

e and f Seedlings were treated 10^{-6} M (e) or 10^{-5} M (f) FUdR for 2 hr.

(e) After 4 hr recovery period, two chromatid breaks (➤) were observed.

(f) A metaphase cell subjected to shuttering of chromosomes at 12 hr after the end of the treatment.

Plate III.

a and b The degree of contraction in metaphase chromosomes were compared in the same magnification between cells which were treated with 0.05% colchicine (a) and with formycin (b).

(a) Seedlings were treated with 0.05% colchicine for 2 hr before fixation.

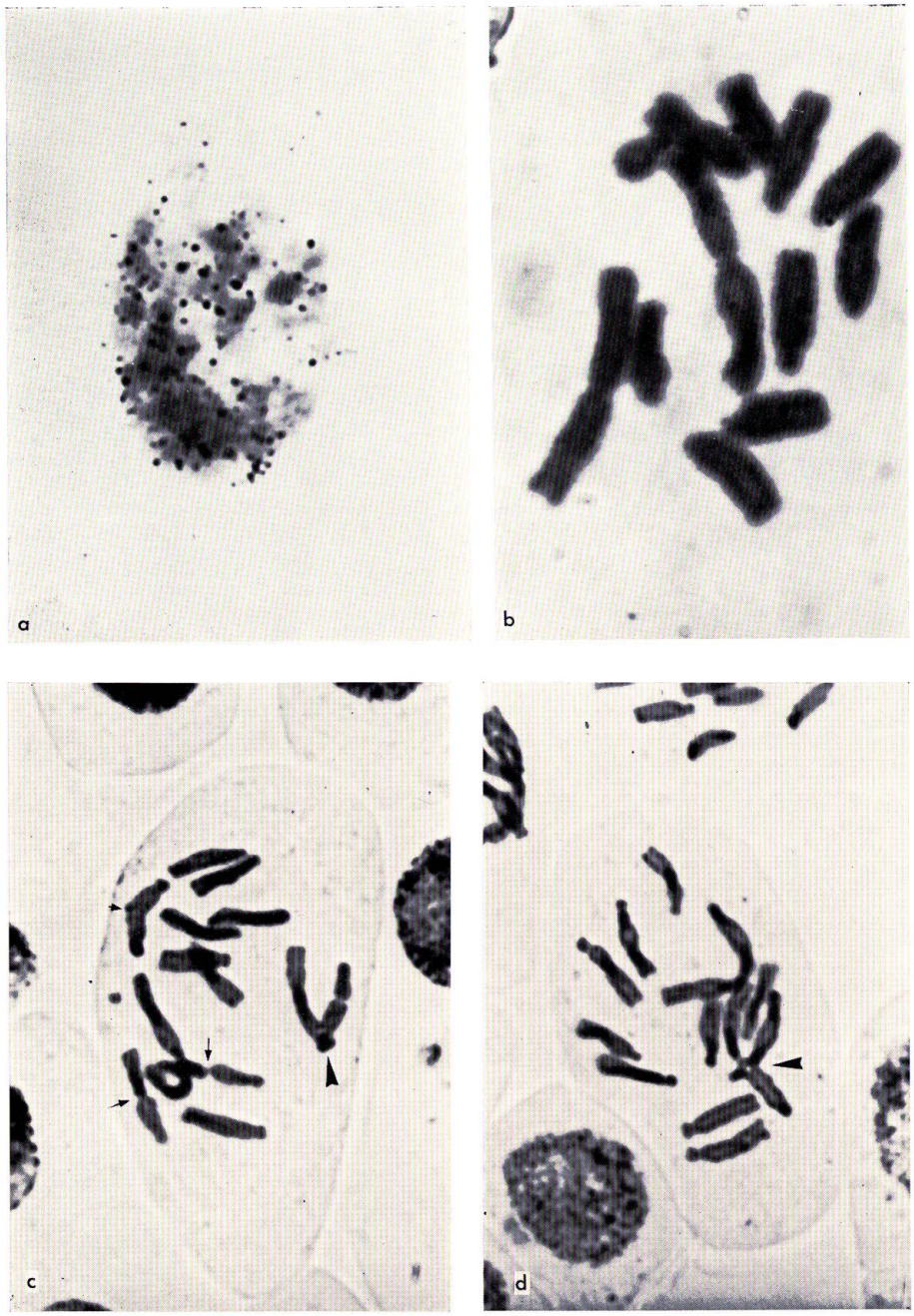
(b) Seedlings were treated with 5×10^{-4} M formycin for 2 hr, and root tips were fixed at 8 hr after the end of treatment. Chromosomes shown in (b) were extremely contracted compared with those in (a).

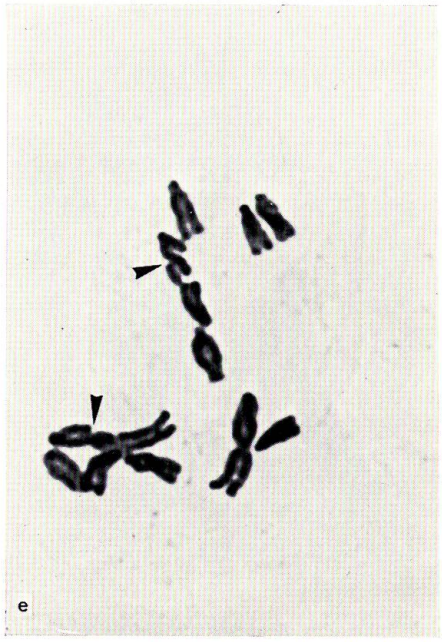
c, d and e Seedlings were treated with 10^{-4} M 3′-AdR for 24 hr (c and d) or for 6 hr (e).

(c) Root tips were fixed immediately after the end of the treatment, and the extreme contraction of metaphase chromosomes and isochromatid breaks in secondary constrictions were observed.

(d) Root tips were fixed immediately after the end of the treatment, and the extreme contraction of anaphase chromosomes and breaks or stretching in the centromeric regions were observed.

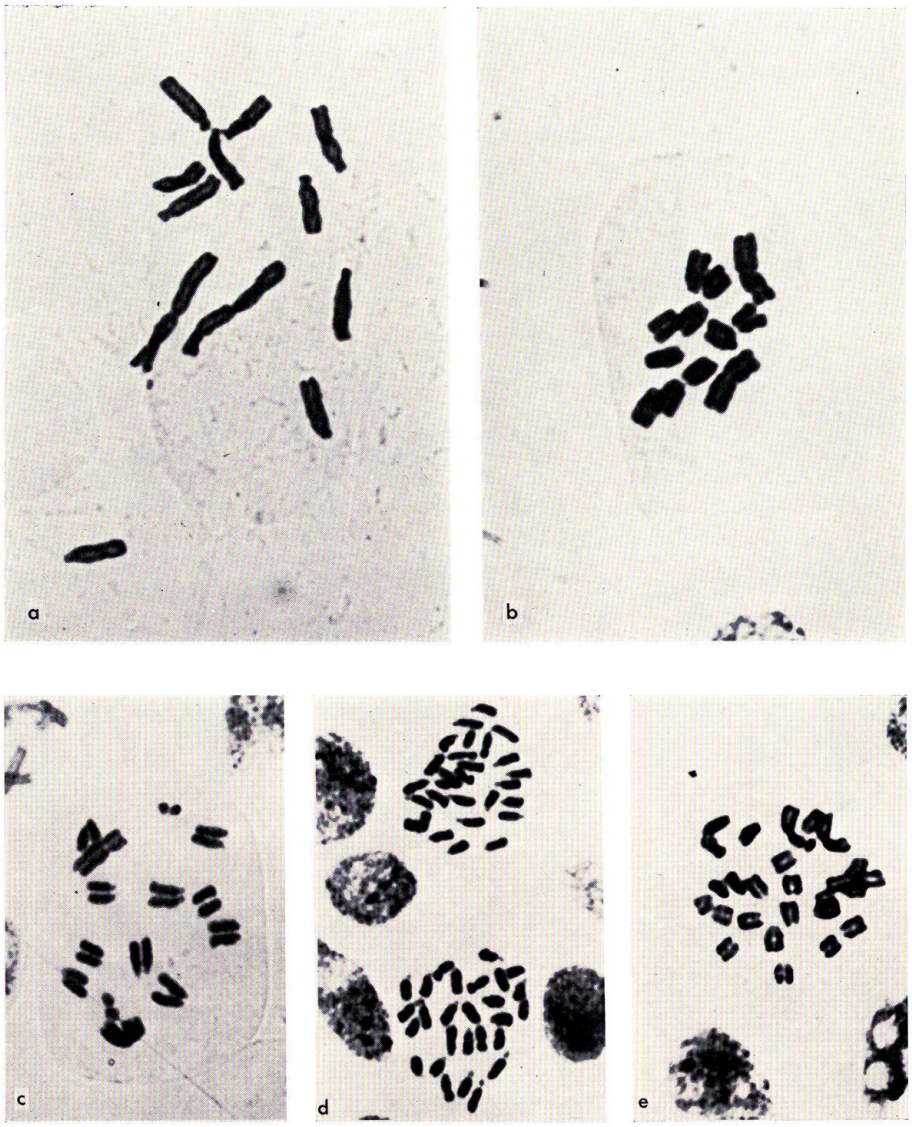
(e) Root tips were fixed at 18 hr of recovery time. A binucleate metaphase cell with extreme contraction of chromosome was observed.


a
b
c
d



e

f


a
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c
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e