INCORPORATION OF C^{14}-LABELED MALEIC HYDRAZIDE INTO THE ROOT-TIP CELLS OF *ALLIUM CERNUUM*, *VICIA FABA*, AND *TRADESCANTIA PALUDOSA*

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ABSTRACT

Allium cernuum, *Vicia faba*, and *Tradescantia paludosa* were treated by root immersion in maleic hydrazide (1 mM/liter) labeled with C^{14} (C^{14}-MH) for 1 hour to determine the location within the cell to which MH moves during various periods of time after treatment. Root tips were fixed 24 hours, 48 hours, 72 hours, and 3 weeks after treatment. Autoradiographs of root tips squashed 24 to 72 hours after fixation showed that C^{14}-MH was distributed throughout the nuclei and was particularly concentrated in the nucleoli. The nucleolar localization of the chemical was transitory, fixations made 3 weeks after treatment showing well labeled nuclei many of which completely lacked label in the nucleoli. The chromosomes seen in mitotic divisions of all three species had the same amount of label in euchromatic as heterochromatic areas. Since the chemical was not accumulated preferentially in heterochromatic areas, it seems likely that the reported specificity of MH for the breakage of heterochromatin can not be due to preferential heterochromatic incorporation.

INTRODUCTION

Following the pioneering studies of Auerbach and Robson (1) on the mutagenic properties of mustard gas, a rather large number of chemicals having mutagenic properties have been discovered. These include such diverse substances as H_2O_2, KCN, formalin, urethane, several phenolic compounds, several heterocyclic compounds, and a number of chelating agents. It appears likely that different mutagens may operate in different ways upon genic material. Some mutagens, for example, may be localized in the cytoplasm and lead indirectly to gene mutation, while others may act directly upon the genes. Some may be most effective upon dividing chromosomes, and others may attack the interphase strands.

The purpose of the study reported here was to determine, by use of an autoradiographic technique (2), the location within the cell to which the heterocyclic mutagen maleic hydrazide (MH) moves during various periods of time after treatment. The chemical is known from the work of McLeish (3, 4), Kihlman (5), and Graf (6) to be a powerful agent for breakage of plant chromosomes. According to these authors, MH breaks the chromosomes specifically in heterochromatic areas. Kihlman (7) has presented indirect evidence leading to the conclusion that the MH breaks chromosomes only of cells that are in division at the time of treatment and cannot get past the nuclear envelope of the interphase cell.

MATERIALS AND METHODS

Allium cernuum, collected at Spring Creek, Center County, Pennsylvania, and *Tradescantia paludosa* (Sax's clone number 3) were treated as mature plants, while *Vicia faba* plants were treated as seedlings (seed supplied by W. Atlee Burpee of Philadelphia).

These three species were chosen because they differ in their heterochromatic content, the nuclei of *Vicia faba* having clear heterochromatin (8), while those of *Allium cernuum* and *Tradescantia paludosa* are euchromatic throughout.

The plants were treated by root immersion in a solution containing 1 millimole per liter of MH labelled with C^{14} (specific activity of 0.1 microcuries per millimole) for 1 hour. The C^{14}-MH was supplied by Naugatuck Chemical Co., Naugatuck, Connecticut, through the courtesy of Dr. J. Zukel. Root tips were fixed at intervals of 24 hours, 48 hours, 72 hours, and 3 weeks after treatment. Before fixation the root tips were pretreated with 8-hydroxyquinoline (Ortho) at a concentration of 0.278 gm/cc for 3 hours, according to the technique of Tjio and Levan (9), to obtain a maximum spread of chromosomes in mitotic figures. The tips were fixed in Carnoy's solution and stored in a refrigerator until squashed. After hydrolysis in 1 N HCl for 4 minutes at 60°C, the tips were smeared in propio-orcein. The coverslips were then removed and the slides were passed through the alcohol series to water. Autoradiographic stripping film from Kodak Limited, England (AR-10) was applied, and the preparations were exposed for 6 months in light-proof plastic boxes containing silica gel in a desiccator under refrigeration. The preparations were developed in Kodak D-19, fixed with Kodak F-6 (1 part F-6 to 2 parts of water), washed in running water for 2 hours, and finally air dried. Immersion oil was used between the film and the coverslip.

RESULTS

C^{14} was predominantly incorporated into nuclei. Some cytoplasmic incorporation was observed in all root-tip squashes, but by comparison with nuclear incorporation the labeling in most cases was without a definite identifiable pattern. Four rather separate categories of nuclei could be identified:

(*a*) Nuclei having only a trace of label or lacking identifiable label.

(*b*) Uniformly labeled nuclei, the label varying from well scattered to comparatively densely concentrated particles (Fig. 1).

(*c*) Nuclei showing predominantly nucleolar localization of the label (Figs. 2 to 5, 7, 8, and 28). Propio-orcein selectively stains chromatin,

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FIGURES 1 TO 8

Radioautographs of root tips fixed at various times after treatment with C^{14}-labeled maleic hydrazide (1 mM/liter) and smeared in propio-orcein. × 1800.

FIGURES 1 AND 2

Allium cernuum tips fixed 24 hours after treatment.

FIGURE 3

Allium cernuum tip fixed 48 hours after treatment.

FIGURE 4

Allium cernuum tip fixed 72 hours after treatment.

FIGURE 5

Tradescantia paludosa tip fixed 24 hours after treatment.

FIGURE 6

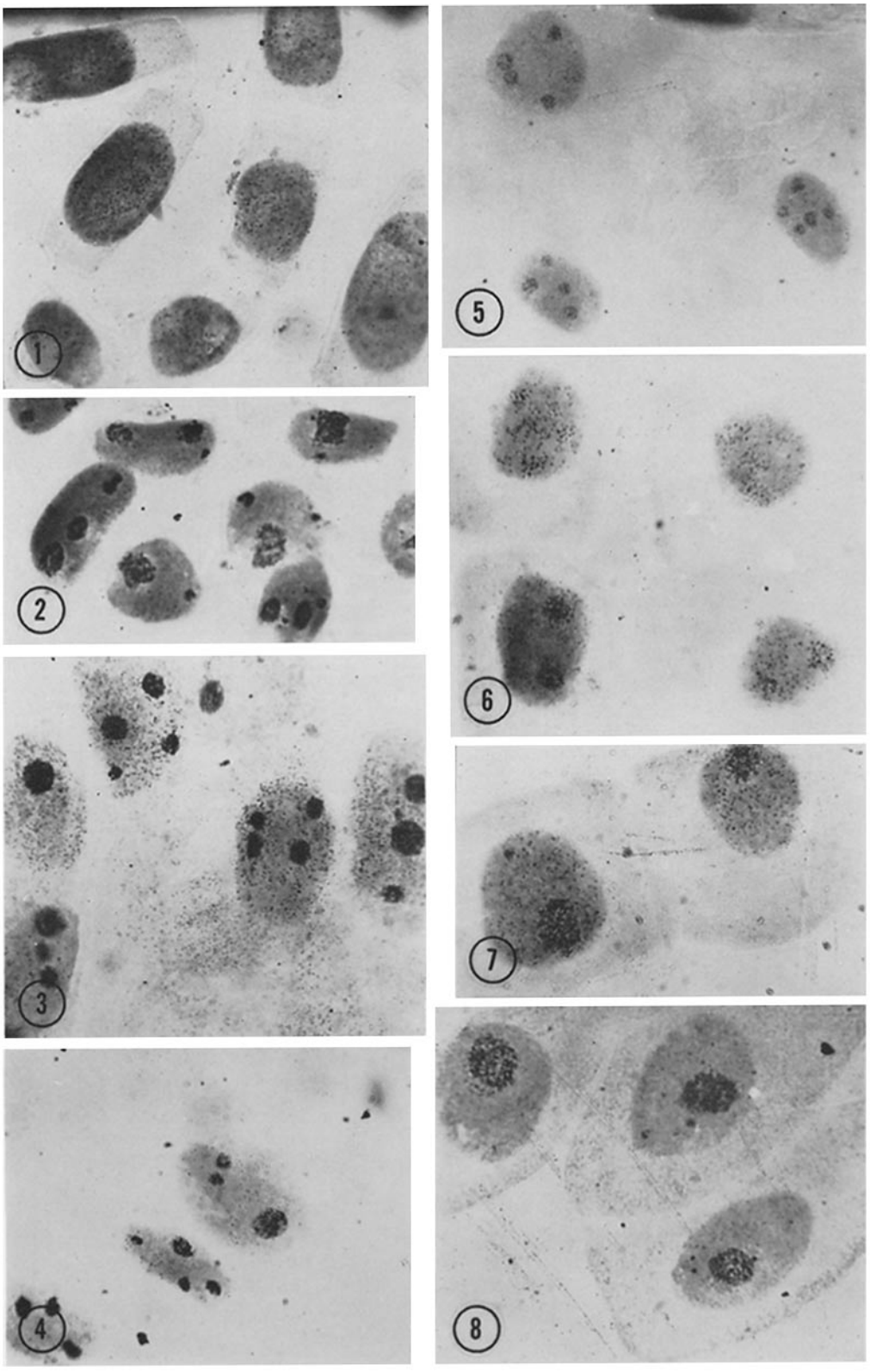
Vicia faba tip fixed 24 hours after treatment.

FIGURE 7

Vicia faba tip fixed 48 hours after treatment.

FIGURE 8

Vicia faba tip fixed 72 hours after treatment.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but not nucleoli, so that nucleoli of nuclei stained by this procedure appear as empty holes. The appearance of such unstained nucleoli can be judged from Figs. 9, 14 to 16, and 19. A dark appearance of nucleoli, therefore, results entirely from the presence of label and not from the presence of the stain. While the categories of labeling were generally distinct, there were some transitional forms occasionally observed. An example of this, in Fig. 6, shows gradations in labeling from uniformly labeled nuclei to nuclei having nucleolar localization of the label.

(*d*) Nuclei predominantly with non-nucleolar label (Figs. 9, 14 to 16, and 19).

Table I shows the frequency of these four categories of C^{14}-labeled nuclei in the root tips of *Allium cernuum*, *Vicia faba*, and *Tradescantia paludosa*. One hundred nuclei of each region of each slide were scored so that the numbers of nuclei listed in each category are the actual numbers observed, and are also the percentage figures. Several regions of each of these smear preparations were separately analyzed to produce the "Region of Slide" figures. The results of analysis of separate regions of the same slide are included to demonstrate the contrast between different populations of cells that presumably originated from different developmental areas of the same tip. In root tips fixed 24 hours after treatment, the great majority of the nuclei appeared to be labeled although some contained only a few grains. Uniformly labeled nuclei and nuclei showing predominantly nucleolar localization of the label occurred in essentially equal numbers in the root-tips fixed 24 to 72 hours after treatment.

Root tips fixed 3 weeks after treatment contrasted with those fixed 24 to 72 hours after treatment in that the label in the nuclei fixed 3 weeks after treatment was not concentrated in the nucleoli. When nucleolar regions were in clear outline and free of superimposed chromatin material, no nucleolar label was observed. Such nuclei were found in the 3-week fixations of all treated plants (Table I). The labeling found in the nucleolar regions of the uniformly labeled nuclei also present in the 3-week fixations could possibly be attributed to labeled chromatin material or to cytoplasmic label lying between the nucleoli and the autoradiographic film.

A concentration of the label in the chromosomal material of nuclei fixed 3 weeks after treatment was evident, for even in early prophases the label outlined the chromatin network (Figs. 10, 15, 20, and 21). The chromosomal material was uniformly labeled in metaphases, anaphases, and telophases of nuclei fixed 3 weeks after treatment (Figs. 11 to 13, 16 to 18, 22, and 23). The mitotic inhibition characteristic of MH (10) results in an almost complete lack of division figures in the first 72 hours after treatment. However, some mitoses have been observed (Figs. 25 to 28) in these early fixations, and without exception the chromosomal material in these figures was also uniformly labeled. All three plant species, irrespective of their differences in content of heterochromatin, showed identical patterns of uniform incorporation of MH along their chromosomes throughout the mitotic cycle.

DISCUSSION

A question of importance in the interpretation of these results is whether maleic hydrazide is stable in plant tissue. The chemical withstands *in vitro* treatment at 200°C with 18 N sulfuric acid or concentrated sodium hydroxide (16), but under combined reduction and hydrolysis the ring opens, yielding quantitative amounts of hydrazine. It has been shown, by use of the Woods method, to be present in plant extracts as long as 8 months after application. Following treatment with C^{14}-MH, Smith *et al.* (17) found that most, if not all, of the C^{14} was recovered in MH. Clagett and Haeseler (oral communication) have shown, however, that about one-fifth of the label recovered 24 hours after treatment of grape leaves occurs in succinic acid or some acid that has similar chromatographic behavior with two different solvent pairs. The remaining recovered label was in MH. The general weight of present evidence, therefore, indicates that MH tends to be stable in plant tissue.

The universal labeling of interphase nuclei observed in early fixations when mitosis was inhibited by MH is evidence that the nuclear membrane offers no resistance to the passage of MH. It is not possible, before analysis has been made of events during the first 24 hours after treatment, to determine whether the MH, once past the nuclear membrane, goes first into nucleoli or other areas of the nuclei. It is clear, however, that the chemical is accumulated early in the nucleoli. This nucleolar localization of label is transitory, since by 3 weeks after treatment the

TABLE I

The Frequency of the Categories of C^{14}-MH-Labeled Nuclei in Vicia faba, Allium cernuum, and Tradescantia paludosa in Root Tip Squashes Taken 24 Hours, 48 Hours, and 3 Weeks after One Initial Treatment with C^{14}-MH (1 mM/liter) for 1 Hour

Plant species	Hours after treatment	Root tip	Region of slide	Nuclear categories			
				Trace label to no label	Uniformly labeled	Predominant nucleolar localization	Predominant nonnucleolar label
Allium cernuum	24	1	a	0	19	81	0
			b	0	50	50	0
			c	0	58	42	0
			d	0	7	93	0
Vicia faba	24	1	a	10	90	0	0
			b	32	68	0	0
			c	40	60	0	0
		2	a	0	15	85	0
			b	0	20	80	0
			c	0	31	69	0
		3	a	0	95	5	0
			b	0	62	38	0
			c	0	76	24	0
Tradescantia paludosa	24	1	a	0	95	5	0
			b	0	98	2	0
			c	0	93	7	0
			d	0	0	100	0
			e	0	25	75	0
Allium cernuum	48	1	a	0	0	100	0
			b	0	0	100	0
			c	0	0	100	0
Vicia faba	48	1	a	0	83	17	0
			b	5	56	44	0
			c	0	80	20	0
		2	a	0	65	35	0
			b	0	78	22	0
			c	0	83	17	0
Allium cernuum	72	1	a	0	53	47	0
			b	0	55	45	0
			c	0	17	83	0
			d	0	83	17	0
		2	a	0	56	44	0
			b	0	49	51	0
			c	0	58	42	0
Vicia faba	72	1	a	0	42	58	0
			b	0	28	72	0
			c	0	60	40	0
Allium cernuum	3 weeks	1	a	0	84	0	16
			b	0	95	0	5
			c	0	85	0	15
Vicia faba	3 weeks	1	a	0	64	0	36
			b	0	69	0	31
			c	0	72	0	28
		2	a	0	74	0	26
			b	0	79	0	21
			c	0	81	0	19
Tradescantia paludosa	3 weeks	1	a	0	60	0	40
			b	0	65	0	35
			c	0	80	0	20

chromatin material is labeled but there is no concentration of label in nucleolar regions. It would not be possible to tell whether the nucleolar label disappears during an interphase stage or following a mitotic division, since all of the cells of the fixation made 3 weeks after treatment have probably gone through one or more mitoses subsequent to labeling. This question can be answered only after study of cells that cannot have gone through mitosis within the 3 week period after treatment. Since the chromosomes of the fixations made 3 weeks after labeling are well labeled, however, it seems likely that chromatin label is not lost during mitotic division. An alternative hypothesis, although a more complex one, is that the labeled MH, or its reaction products, has, after a 3 week period, reached a state of equilibrium during which it moves back and forth between chromatin and cytoplasm.

MH is a structural isomer of the pyrimidine uracil which is a normal component of ribonucleic acid (RNA). Greulach (11) reported in a brief note that the pyrimidines thymine, uracil, and thiouracil appear to have some effects as antagonists of MH in treated plants. He attributed this antagonism to the close structural relationship between these compounds and MH. Recent work by Goldstein and Micou (12) provides evidence that nuclear RNA is synthesized at many chromosomal loci and is very rapidly transported to nucleoli where some other activity takes place at a slower rate. This nucleolar activity is assumed to be a conjugation of RNA with protein. Such a hypothesis has also been suggested by Woods and Taylor (13) and Woods (15). The nuclear incorporation of C^{14}-MH may follow this assumed pathway of RNA synthesis, the MH taking the place normally held by uracil on the RNA. The end product would be expected to be a modified RNA. It is conceivable that the heavily labeled chromosomal material characteristic of the nuclei fixed 3 weeks after treatment is indicative of the presence of such a modified RNA in a conjugated ribonucleoprotein. One piece of evidence that disagrees with this hypothesis is the fact that hydrolysis with HCl, a procedure commonly used for removal of RNA, was used in preparation of these root tips. The period of hydrolysis was a relatively short one (4 minutes) and whole root tips rather than sections were treated, so that the bulk of the RNA could have been retained in the tips. Studies of the effect of use of enzymatic extraction procedures following incorporation of labeled MH are in progress to determine whether, in fact, the MH is present in a form of RNA.

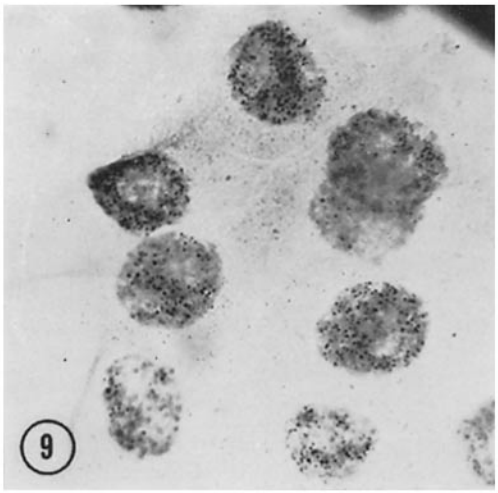
There is no indication of a concentration of MH in the known heterochromatic areas of *Vicia faba*. Even though MH has been demonstrated (3–6) to have a selective breaking action on heterochromatin, the labeling of euchromatin and heterochromatin of *Vicia faba* is the same and does not differ from the chromosomal labeling of the other two species though they lack visible heterochromatin. Darlington and McLeish (14), McLeish (3, 4), and Kihlman (5) all reported that there is a high incidence of breaks in the proximal heterochromatic block of the nucleolar arms of the long chromosomes of MH-treated *Vicia faba*. The breakdage frequency in the heterochromatic area of the nucleolar arm is as much as 3 to 10 times greater than that of other heterochromatic areas. Since in the interphase nucleus the nucleolar organizer region is attached to the nucleolus, this would necessarily mean that the proximal heterochromatic areas lie closer to the nucleoli than do the other heterochromatic blocks. It is conceivable that this high breakage frequency of the proximal heterochromatic area of the nucleolar organizing arms is due to the proximity to the observed concentrations of MH in the nucleoli of many nuclei 24 to 72 hours after treatment. It would appear, in any case, that the selective

FIGURES 9 TO 13

Radioautographs of *Allium cernuum* root tip squashes fixed 3 weeks after treatment with C^{14}-MH (1 mM/liter). × 1800.

FIGURES 14 TO 18

Radioautographs of *Vicia faba* root tip squashes fixed 3 weeks after treatment with C^{14}-MH (1mM/liter) for 1 hour. × 1800.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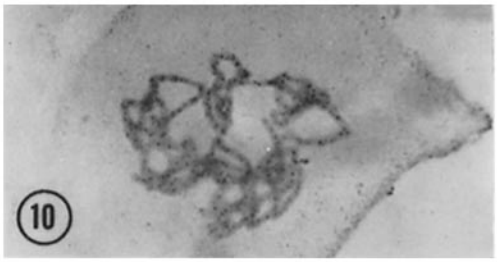
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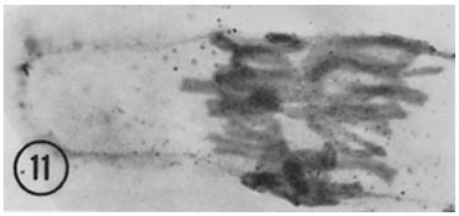
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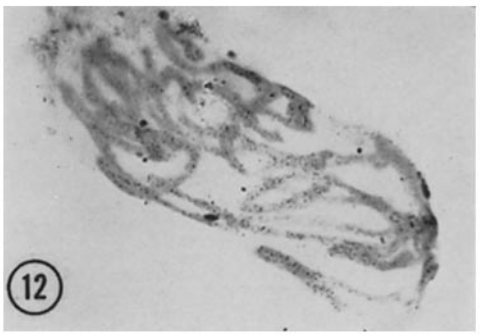
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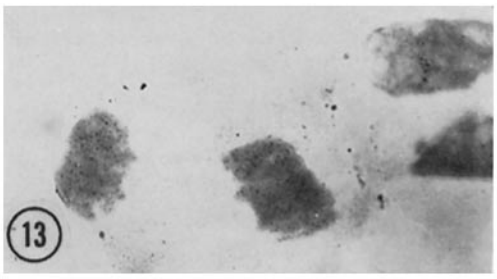
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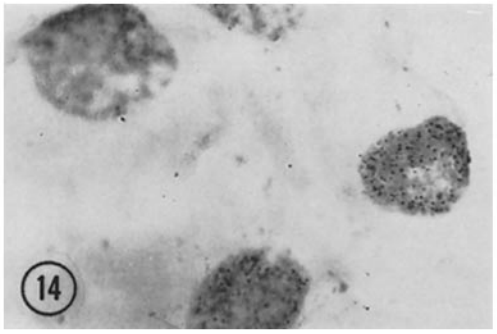
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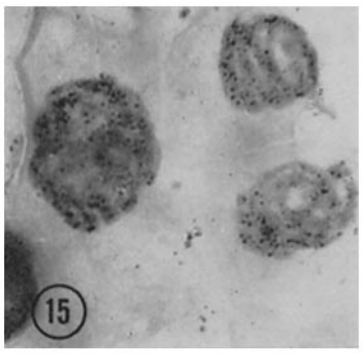
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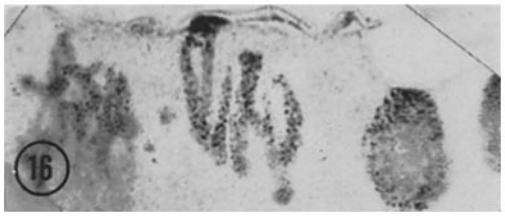
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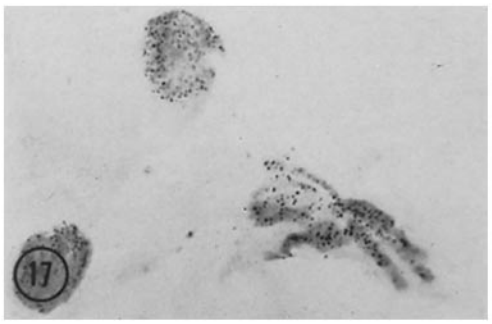
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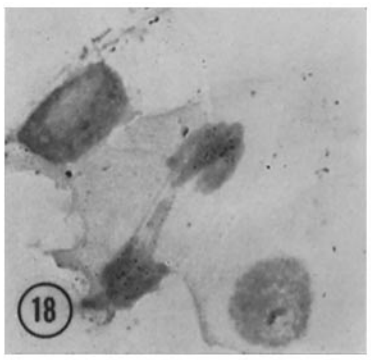
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breakage of heterochromatin by MH does not result from a direct accumulation of the chemical on heterochromatic loci of the chromosomes but from some more indirect cause.

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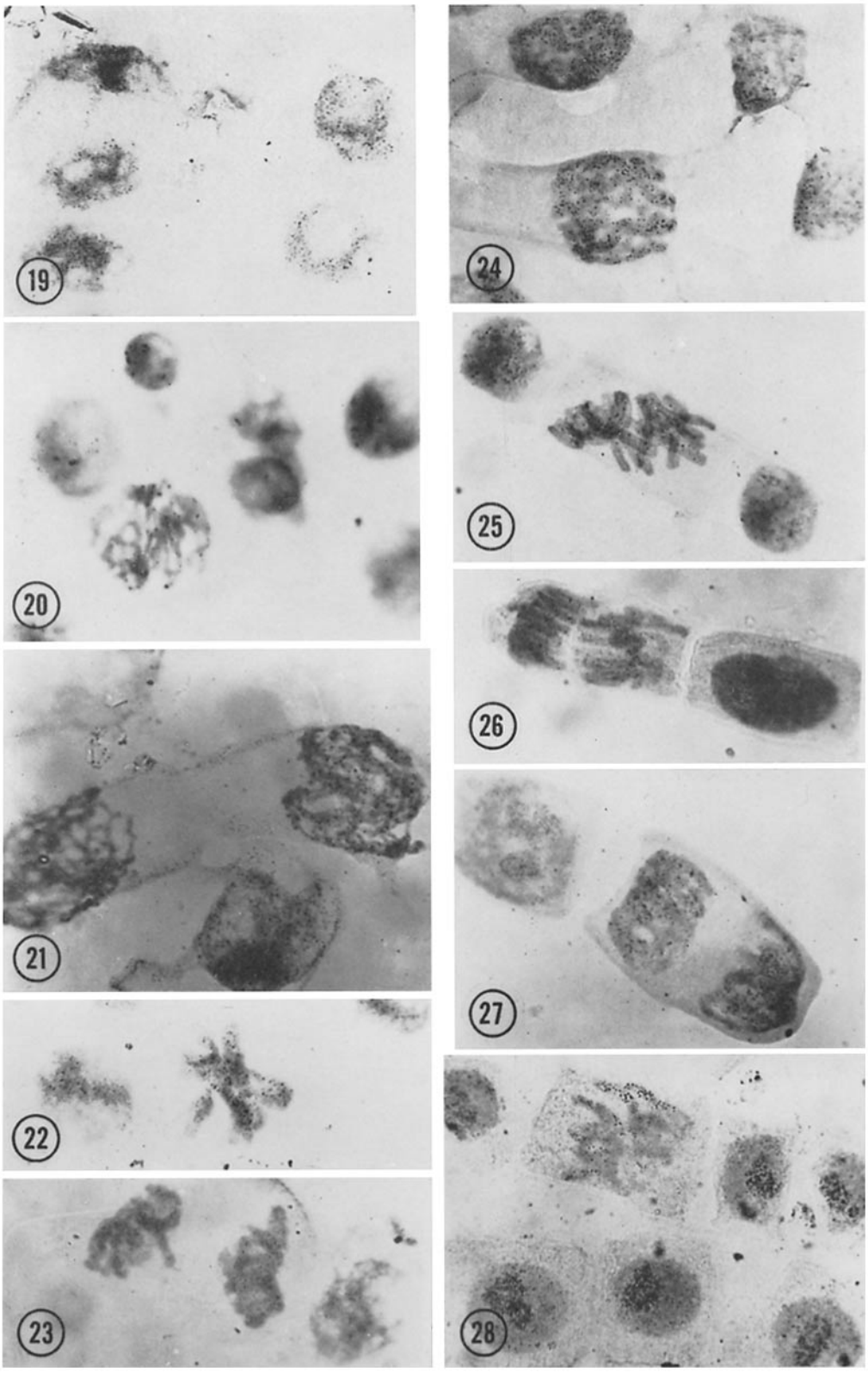
FIGURES 19 TO 23

Radioautographs of *Tradescantia paludosa* root tip squashes fixed 3 weeks after treatment with C^{14}-MH (1 mM/liter) for 1 hour. Stained in propio-orcein. × 1800.

FIGURES 24 TO 28

Radioautographs of *Allium cernuum* root tip squashes fixed 24 hours after treatment with C^{14}-MH (1 mM/liter) for 1 hour. Stained in propio-orcein. × 1800.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