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CHROMOSOME BREAKAGE BY ETHYLENIMINES AND RELATED COMPOUNDS[1])

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Primary roots of *Pisum sativum* and *Vicia faba* were treated for ½ hour with several ethylenimines and related compounds. Root samples were removed periodically for several mitotic cycles, and squash preparations were made and scored for anaphase damage as indicated by bridges and fragments. It was observed that maximum damage did not appear until after a delay which varied from 2 to 3 mitotic cycles after treatment, then dropped off in some cases, and persisted in others. The mitotic delay was estimated to be about 3 hours in *Pisum*, and 6–7 hours in *Vicia*. This delay is attributed to one or more of the following: (1) the number of strands in a chromosome, (2) the unit of breakage, and (3) the type of assortment of the chromatids, whether random or not. Some chemicals were mutagenic, although they did not induce any detectable chromosome damage. Experiments in which 5-amino-uracil was used did not indicate that any particular stage of the mitotic cycle was especially susceptible to damage. Preliminary experiments with DNP show that ATP is required either for uptake or site binding of the chemicals before actual breakage.

Introduction

Many chemicals are known to cause chromosome breakage. An extensive review has been published by KIHLMAN (1966) who points out that the bulk of active agents seem to interfere one way or another in DNA synthesis. KIHLMAN also makes a distinction between agents with immediate effects and those with delayed effects. In the present

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work we classify as a delayed type any agent which shows a maximum effect more than one full mitotic cycle after exposure, taking into account any delaying effect in cycle time, that treatment may induce.

The matter of chemically-induced chromosome damage has been given immediacy by the fact that many such compounds are used or proposed for use in agriculture and medicine. It is also to the point that SAX & SAX (1966) have shown a number of widely used substances, such as alcohol and caffeine, to be as effective as substantial exposure to X-rays.

The present report concerns chromosome breakage as induced by ethylenimines; especially apholate and metepa (Fig. 1). Both are considered as chemosterilants for insect control. Apholate has been shown to be effective in mosquitoes (RAI, 1966) and on barley seeds (WUU & GRANT, 1966). A related compound tretamine (Triethylenemelamine) has been shown to be effective as a chromosome breaker in plant and animal cells as well as to be truly mutagenic (KIHLMAN, 1966; LORKIEWICZ & SZYBALSKI, 1961).

Materials and Methods

Some tests were run on the effects of azirane, tretamine, tepa, metepa, and apholate, as well as on related compounds hempa and ethylenesulfide (Fig. 1).

The Pisum system: Routine tests were run on the *Pisum* system as outlined by WILSON (1965). Essentially, the system consists of germinating the peas until the primary root is about 2 cm, suspending the seedlings by paraffin-coated wire mesh with the root in a balanced salt solution at pH about 5.7, and a temperature of 22.5°C. The chemical to be tested was added to the salt solution. Exposure was for ½ hour, after which the seedlings were well washed and returned to nutreint, with samples for cytological examination being taken at predetermined times. Most analyses were for mitotic index (M.I., the number of dividing cells per a total of 1,000 cells counted) and anaphase damage, as indicated by bridges and fragments. Some studies were made of metaphase rearrangements by treating for 45 minutes with ruelene to scatter the chromosomes.

The Vicia system: For certain studies, most particularly those concerned with types of rearrangements, seedlings of *Vicia faba* were

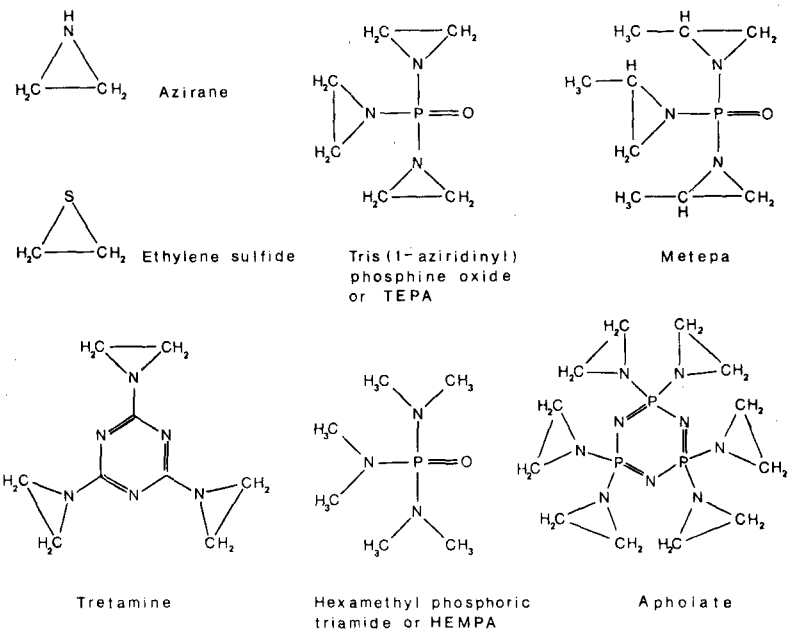

Figure 1. Structural formulas of ethylenimines and related compounds used in this study.

used. Handling was in all respects identical to that used for *Pisum*. In order to scatter the chromosomes for metaphase analysis, seedlings were soaked for 1 hour in paradichlorobenzene before fixation.

Cycle time measurement: Relative mitotic cycle times were measured by exposing roots to about 75 ppm colchicine continiously, and determining the time of appearance of polyploid divisions. Differences between treated and control were taken as indicating difference in cycle time. The method has been compared to the polyploid population system of VAN'T HOF et. al. (1963), and to systems using tritiated thymidine, and was found to be at least as reliable (BEKKEN, 1966, unpublished).

The 5-amino-uracil (5-AU) system: When roots are treated with 5-aminouracil, cells pile up in the G_1 segment of interphase. When released from treatment, these stalled cells proceed on into S, G_2, and mitosis in synchrony. This synchrony is not, however, notably retained, since by the end of the next cycle the mitotic index is not

detectably higher than normal. If pea roots are treated for 8 hours with 150 ppm of 5-AU, the mitotic index falls to a low level at about 8 hours, begins to rise again at 12 hours, and reaches a peak at 15–18 hours. It can be assumed (and some treatments with tritiated thymidine have tended to confirm the assumption) that at 7–8 hours most cells in the cycle are in G_1 S, at 12–13 hours they are in G_2, and at 15–18 hours they are in mitosis. On this basis we exposed such 5-AU treated pea roots to 3.1×10^{-3} molar apholate for $\frac{1}{2}$ hour at $7\frac{1}{2}$ hours, 10 hours, 12 hours, and 15 hours, and determined time and degree of maximum damaged anaphases.

Hempa and apholate were both tested for mutagenic capacity. The method was to dip Late-third instar larvae or prepupae of *Drosophila melanogaster* Oregon R wild strain in 1% solution for 30–40 seconds, cross treated males to Muller-5 females, and raise an F_2 from pair matings. F_2 vials containing no red-eyed males were considered positive, and were always retested.

Results

General: All of the true ethylenimines caused substantial chromosome damage, but neither of the related compounds hempa and ethylenesulfide were effective at non-toxic levels. On a molar basis the order of effectiveness (in terms of maximum damage as scored at anaphase) was found to be from least to most effective: azirane, tepa, metepa, apholate, and tretamine. In all cases the effect was of the delayed type; that is, a maximum damage index was not found until after at least one mitotic cycle. In most cases in peas, maximum damage was at about 27 hours after treatment, although there were exceptions (as noted later).

Although the numbers to date are small, both hempa (which was cytologically ineffective in chromosome breakage) and apholate (which was very effective cytologically) gave positive results when checked by the Muller-5 system for mutagenicity. In the former case we found 3 positives out of a total of 153 X-chromosomes treated, and in the latter, 20 out of 485, which are equivalent to 1.95% and 3.9%, respectively. Control value for the Oregon R stock is 0.0974% (SPENCER & STERN, 1948).

Apholate: a fairly elaborate study has been made of the effects of

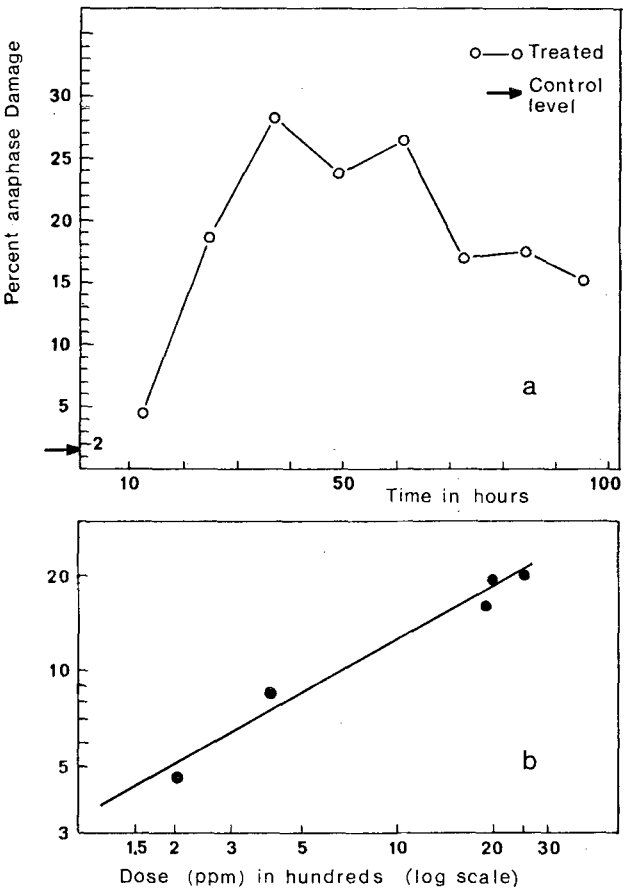

Figure 2. – a, Anaphase damage in peas treated for ½ hour with 0.003 M apholate. 3rd cycle maximum and persistent effect. – b, Dose effect curve using apholate (log scale).

apholate on pea roots. The results are in some ways both surprising and confusing. On some occasions the maximum damage was not reached until about 39 hours after treatment, which time span is equal to about three full mitotic cycles. Furthermore, the damage level remained high until 95 hours or longer (Fig. 2a). In these experiments there was little sign of toxicity, and the mitotic index remained at approximately control value. In other cases, maximum damage was reached at about 27 hours, and fell sharply thereafter, though it

TABLE 1a

ANAPHASE DAMAGE AND MITOTIC INDEX (MI) IN PEA ROOTS TREATED WITH 150 PPM OF 5-AMINO-URACIL FOR 7½ HORS, FOLLOWED BY 0.003 M APHOLATE FOR ½ HOUR OR TREATED IN G_1S

Hours	No. of anas. counted	No. of damaged anas.	% of damage	Av. M.I.
7½	—	—	—	14
12½	—	—	—	38
15	600	29	5.0	80
16	800	46	5.8	123
17	600	20	3.3	104
18	800	49	6.1	99
19	600	10	1.7	76
20	800	16	2.0	70
21	800	24	3.0	55
22	400	12	3.0	50
24	600	21	3.5	50
27	800	82	10.2	57
32	400	36	9.0	60
34	600	75	12.5	73
36	400	52	13.0	43
39	200	30	15.0	32
51	200	22	11.0	57

remained generally above control level for many hours. In these cases mitotic activity, as indicated by mitotic index, tended to be erratic. Each time it was measured, mitotic cycle time was increased by 2–3 hours for the first cycle after treatment, and remained normal for subsequent cycles.

As noted, use of 5-AU allows treatment of cells at G_1 S; S; S G_2; and mitosis. In all cases (Tables 1a, 1b, 1c, and 1d), the maximum damage appeared at a time corresponding to the second mitosis after treatment, and persisted for some hours thereafter. Both amount and time of damage appeared to be relatively independent of time of treatment. Such differences as did show are considered insignificant.

Some studies of dose vs. maximum effect were also made. In general, when log of dose is plotted against log of concentration, a straight line results (Fig. 2b).

TABLE 1b

ANAPHASE DAMAGE AND MITOTIC INDEX (MI) IN PEA ROOTS TREATED WITH 150PPM OF 5-AMINO-URACIL FOR 8 HOURS, FOLLOWED BY 0.003 M APHOLATE FOR ½ HOUR AT 10½ HOURS OR TREATED IN 'S' STAGE

Hours	No. of anas. counted	No. of damaged anas.	% of damage	Av. M.I.
8	—	—	—	6
10	—	—	—	4
12	—	—	—	6
15	1000	18	1.8	207
17	600	17	2.8	140
19	800	22	2.8	43
21	400	21	5.1	59
24	400	23	5.7	18
26	200	17	8.5	40
27	600	58	9.7	54
30	400	55	13.7	44
32	600	77	12.9	57
39	600	77	12.9	50
42	400	51	12.7	42
51	400	36	9.0	23

Metepa: Similar studies were made of the effects of metepa on pea roots with virtually identical results. Some experiments showed a maximum damage at 39 hours, and others at about 27 hours (Fig. 3a and b). In some cases damage persisted at a high level, and in others it fell off, although generally it remained above control level for long periods. Mitotic indices as in the case of apholate were constant in the one case, and erratic in the other.

Somewhat similar studies were made of the effects of metepa on chromosomes in the meristem of the primary root of *Vicia faba* seedlings. Again, the maximum anaphase damage did not appear until about 27 hours, which is more than one cycle after treatment, even when we take into account that metepa appears to delay the first cycle 6–7 hours; i.e.; control cycle time is about 17 hours, treated 23–24 hours. Subsequent cycles, as in the case of pea roots, are not altered to any detectible degree.

Both chromosome and chromatid breaks and rearrangements

TABLE 1c

ANAPHASE DAMAGE AND MITOTIC INDEX (MI) IN PEA ROOTS TREATED WITH 100 PPM of 5-AMINO-URACIL FOR 8 HOURS, FOLLOWED BY 0.003 M APHOLATE FOR ½ HOUR AT 12½ HOURS OR TREATED IN SG_2

Hours	No. of anas. counted	No. of damaged anas.	% of damage	Av. M.I.
7½	—	—	—	18
12½	—	—	—	28
15	600	7	1.1	110
16	400	11	2.7	153
17	400	5	1.3	141
18	400	8	2.0	98
20	800	6	0.7	75
21	600	5	0.8	51
22	400	3	0.7	31
24	600	30	5.0	53
27	600	60	10.0	36
32	600	60	10.0	45
34	400	45	11.2	100
36	400	46	11.5	47
39	400	52	13.0	70
51	1000	101	10.1	60

TABLE 1d

ANAPHASE DAMAGE AND MITOTIC INDEX (MI) IN PEA ROOTS TREATED WITH 150 PPM OF 5-AMINO-URACIL FOR 8 HOURS, FOLLOWED BY 0.003 M APHOLATE FOR ½ HOUR AT 15 HOURS OR TREATED IN M

Hours	No. of anas. counted	No. of damaged anas.	% of damage	Av. M.I.
8	—	—	—	1
12	—	—	—	1
15	400	6	1.5	266
16	600	8	1.3	188
17	200	1	0.5	185
30	1000	58	5.8	63
31	1000	117	11.7	49
32	1000	98	9.8	48
42	800	82	10.2	44

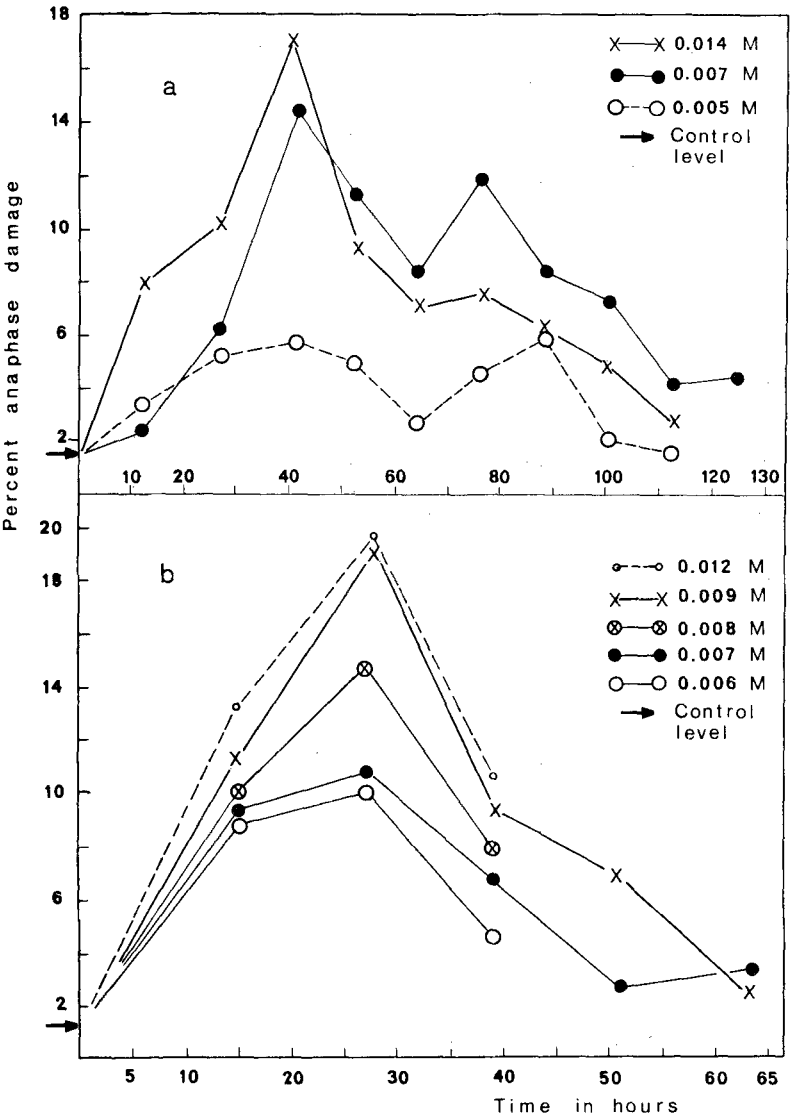

Figure 3. – a, Anaphase damage in peas treated with metepa for ½ hour. Maximum damage at the 3rd cycle after treatment. – b, Anaphase damage in peas treated with metepa for ½ hour. Maximum damage a the 3rd cycle after treatment.

TABLE 2

PERCENT ABERRATIONS OF VARIOUS KINDS IN *Vicia faba* INDUCED BY 0.233×10^{-2} M METEPA TREATMENT

Hours	Chromatid damage:			Chromosome damage:				Total:	
	Break	Exchange	Total %	Break	Exchange	Fragment *	Total %	Cells counted	Damage %
0	—	—	—	1	—	—	1	100	1
2½	1	—	1	—	—	—	—	100	1
6	—	—	—	3	—	—	6	50	6
10	—	2	2	11	—	—	11	100	13
12	—	—	—	8	—	—	8	100	8
14	3	1	4	14	1	—	15	100	19
25	14	8	27.5	22	2	—	30	80	57.5
28	20	12	32	25	6	—	31	100	63
45	14	13	27	14	10	—	24	100	51
61	2	1	20	8	6	17	14	100	34
72	2	—	9	6	—	7	6	100	15

* Centric fragments.

showed a maximum at about the same time (see Table 2), although significant chromosome damage showed up somewhat earlier. Chromatid deletions continued to appear up to at least 72 hours. Some chromosome fragments were also found at late hours, although at least some of these represent a second cycle of damaged cells as indicated by deficiencies, and were presumably originally chromatid events. The damage consisted largely of chromosome and chromatid fragments, and chromatid exchanges (Figures 4, 5, and 6). The chromosome exchanges seem to have been secondarily derived from dicentric chromatids going through more than one cycle.

Some preliminary studies have been made of the effects of 2–4 Dinitrophenol (DNP) on the effect of apholate on pea roots. So far the evidence is that DNP inhibits uptake. When roots were treated first for 15 minutes with 10 ppm DNP and then for ½ hour with 3000 ppm apholate, damage was found to be only slightly above control level. Treatment with DNP after apholate exposure produced results not significantly different from treatment with apholate alone.

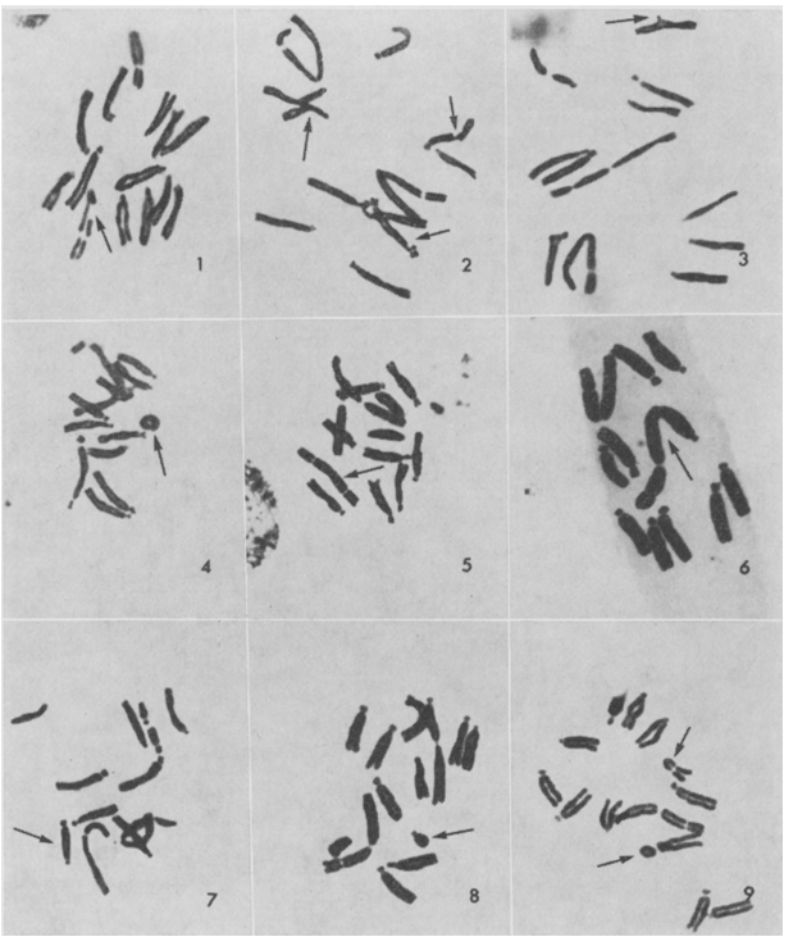

Figure 4. Types of aberrations induced by metepa on *Vicia faba*. 1, 2, 3, chromatid breaks. – 4, ring chromosome. – 5, 6, 7, chromosome exchanges (dicentric chromosomes). – 8, 9, isochromatid rejoinings.

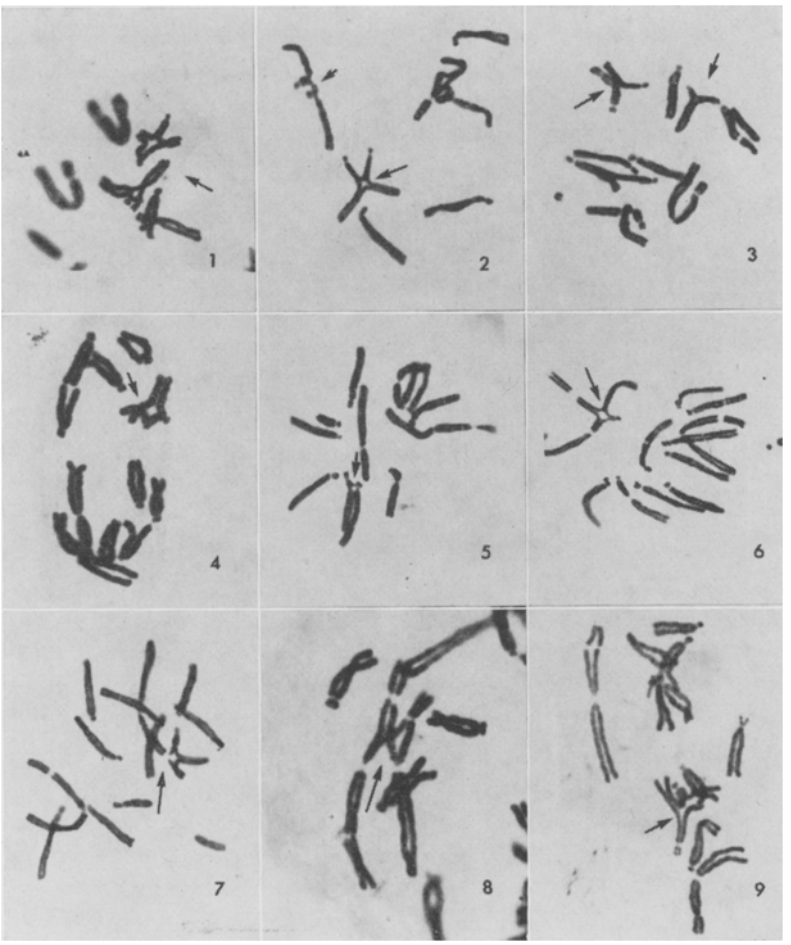

Figure 5. Types of exchanges induced by metepa on *Vicia faba*. 1, 8, longitudinal chromated exchange involving two 'S' chromosomes and two 'M' chromosomes. – 2, 3, 4, 5, 6, 7, transverse chromatid exchanges. – 9, a complex chromatid exchange involving three chromosomes.

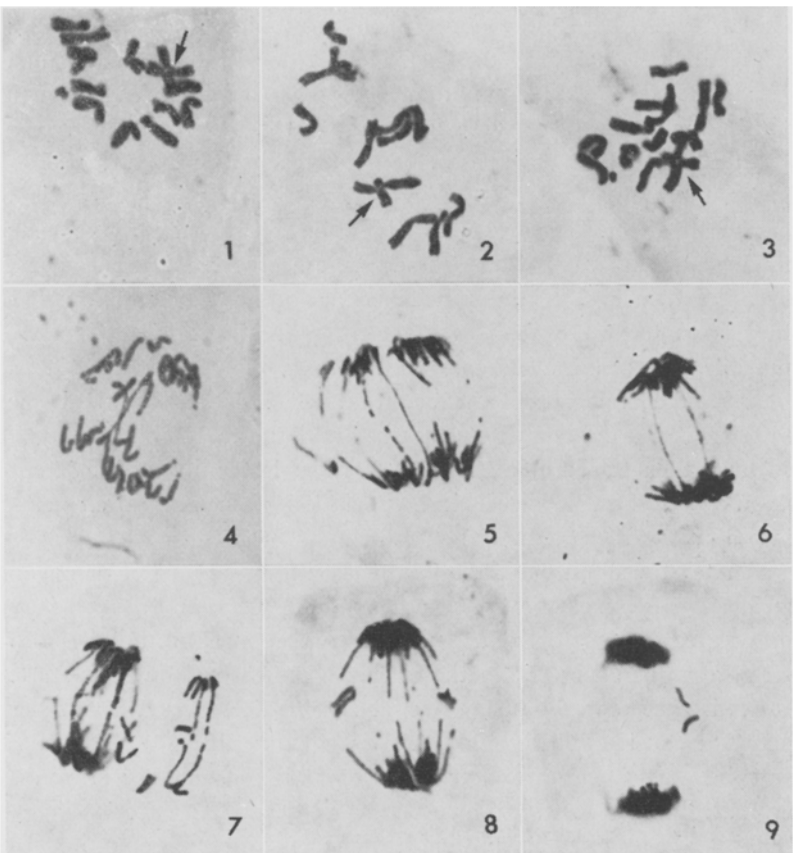

Figure 6. Metaphase and anaphase damages induced by apholate in peas. 1, 2, 3, chromatid exchanges. – 4, x-shaped anaphase bridges. – 5, anaphase bridge and fragment. – 6, a double bridge. – 7, bridges and fragments. – 8, u-shaped fragment. – 9, telophase with two chromatid fragments.

Discussion

Delayed effect: If a peak effect does not appear until after a period equal to one mitotic cycle, taking due cognizance of any delay induced by treatment, it may be said to be a delayed effect. In *Pisum*, if the peak effect appears after 15 hours, it probably should be considered delayed as with metepa and apholate. At any rate, in peas the first mitotic cycle after treatment is increased by not more than 3 hours, and subsequent cycles not at all. Delay itself may have a number of

explanations. Some of the more obvious possibilities are the following:

(1) Unusual delay in damaged cells reaching division. Against this is the fact that we found damaged cells in *Vicia* coming through to division as fast as non-damaged ones.

(2) If the bulk of damage was done at the subchromatid level or lower, there might be a delay before the original damage became a chromatid effect. There are two major objections to this; (a) the delay in chromosome breaks which should have been deleted as chromatid events earlier, and (b) the unlikelihood of breaks remaining open as they must, to form exchanges at the chromatid level. It is, of course, possible that the initial damage is not a break, but a point of weakness.

It has generally been assumed that the half chromatid of one division is the chromatid of the next, and the fourth chromatid, if such exists, is the chromatid of two divisions in the future. We do not know for certain, however, that association of the several elements into a chromatid is entirely non-random.

Whatever the reason, there is a delay in appearance of damage which is more than one cycle duration, and which on occasion may be of greater length. Such a delay is not explicable at present in terms of any known cell kinetics or element of chromosome structure.

Persistence of the damage over a considerable period is another point of considerable interest, and is equally difficult to explain. In some experiments we have found damage considerably in excess of control level; at times corresponding to 10 or 12 mitotic cycles. This may be ascribed to multiple strandedness of the chromosome, to random assortment of chromosome elements at each division, or to a combination of both. Since WUU & GRANT (1966) found significant damage in the next generation in barley, deviation from preferential assortment of chromosome elements seems most plausible. On this basis, damage at the half chromatid level or lower has a possibility of being hidden indefinitely.

The 5-amino-uracil technique allowed us to treat cells mainly in G_1S, S, G_2, and M, and to compare the time and degree of maximum effect. The results certainly do not suggest any particular period of high or low susceptibility. It may be, however, that there was always enough of the chemical present in the cell to do maximum damage whenever the cell reached the critical stage. Table 3 gives the hours of treatment and the stages passed through before maximum damage.

TABLE 3

TREATMENT TIMES WITH APHOLATE AND SEQUENCES OF THE MITOTIC CYCLE THE CELLS PASSED THROUGH BEFORE MAXIMUM ANAPHASE DAMAGE WAS OBTAINED

Treatment times of apholate after the beginning of 5-AU treatment	Stages of the cycle cells passed through before maximum anaphase damage was obtained
$7\frac{1}{2}$	$S - G_2 - M - G_1 - S - G_2 - M$
10	$G_2 - M - G_1 - S - G_2 - M$
$12\frac{1}{2}$	$G_2 - M - G_1 - S - G_2 - M$
15	$M - G_1 - S - G_2 - M$

We are forced to conclude that for maximum damage to appear, a period equal to at least one full mitotic cycle must pass, and presumably affected cells must go through all the stages of that cycle. Clearly, however, the delay may be longer. Some damage does show up prior to one full average cycle, but this may represent cells which pass through rapidly. Under control conditions, for example, some cells do pass through a cycle in as little as 8 hours or less. Our first thought was that damaged cells must pass through the S stage at least once, if not twice, but the evidence from the 5-amino-uracil experiments does not bear out this notion, since the minimum stages appear to be G_1–S–G_2.

Only hempa and apholate were tested for mutagenic capacity by the Muller-5 *Drosophila* system, and both gave positive results. Only the latter, however, did significant damage to the integrity of the chromosome. It seems likely that a chemical which damages chromosomes, and especially when the damage is more or less random, can be counted on to be mutagenic in the strict sense; but lack of "radiomimetic" potential does not rule out mutagenic capacity.

The term "radiomimetic" has been used generally for chemicals causing chromosome fragmentation. The term is not especially apt, since it implies more similarity to the effect of ionizing radiation than is so in the case of the ethylenimines and other like-acting chemicals. For one thing, the delayed effect is markedly different from the almost immediate maximum effect of X-rays. Also, we have seen no indication of configurations suggesting subchromatid exchange (WILSON et. al., 1960) after treatment with the chemicals, while such configurations appeared at 3 hours after X-ray treatment.

Our first impression from apholate-treated peas was that secondary constructions were preferentially, though not exclusively, attacked. To date, however, analysis of *Vicia* data suggest at least nearly random distribution of breaks.

KIHLMAN (1966) points out that most chemical chromosome breakers act by some interference with DNA; either by reacting with precursors, or by interfering more directly in replication. G_1 S stages should, on this view, be the most susceptible, though we have seen little evidence in the case of the ethylenimines (including tretamine) which is supposed, according to LORKIEWICZ & SZYBALSKI (1961) to react with phosphorylated thymidylate. At present it seems most likely that the ethylenimines and other alkylating agents may lock into some element of the chromosome at any time, and set up a potential break to be realized at some future and apparently variable time.

From the DNP experiments so far performed, it seems that ATP is not essential in initial damage, but does determine uptake. Whether it is a factor in final breakage or rearrangement, we do not know.

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