STUDIORUM PROGRESSUS

Similar Cytological Effects of Hydroxylamine and 5-FUDR, Agents with Different Modes of Action

An Analysis of Chromosome Breakage

Introduction. For several years attempts have been made to elucidate the mechanisms of action of a variety of radiomimetic agents in the production of chromosome breaks. Recent studies with analogs of nitrogenous bases of DNA and agents which influence DNA metabolism have provided some information regarding the mechanisms of mutation induction. Certain of the chemical agents used produce their effects either by incorporation into the DNA molecule, resulting in base-substitution, or by indirectly effecting a change in the base composition of the DNA molecule. Specific mutations in certain microorganisms have been attributed to changes of this type otherwise referred to as transitions and transversions[1]. As a corollary to observations of mutation induction, cytological effects of such agents might be expected, namely, chromosome breakage and/or mitotic inhibition. Among the several agents used by various investigators, hydroxylamine and 5-fluorodeoxyuridine, each having a metabolic effect quite different from the other, have been found effective in causing chromosome breakage in certain plant and animal tissues.

Hydroxylamine causes inactivation of certain viruses, including T4 bacteriophage, while having no such effect on other viruses[2,3]. Whereas low concentrations of hydroxylamine result in inactivation of T4 phage, high concentrations of the agent are mutagenic[3]. The site of action of hydroxylamine appears to be in DNA at the nitrogenous base cytosine[4], and its reactivity is greater at a slightly acid pH than at a basic pH. In Tobacco Mosaic Virus, however, cytosine is more reactive with hydroxylamine at pH 6 while uracil shows greater reactivity at pH 9[5]. According to FREESE[1], cytosine undergoes a change to a dihydroxylamino compound in the presence of hydroxylamine; at a basic pH, the altered cytosine is partially reverted to cytosine. In terms of mutation production, hydroxylamine treatment results in base-pair transitions.

Treatment with low concentrations of hydroxylamine resulted in significant chromosome breakage in Chinese hamster cells grown *in vitro*[6]. Among the cytological effects observed were multiple constrictions in the chromosomes and chromosomal aberrations. The latter included translocations resulting from both chromatid and chromosome breaks. Analyses of chromosomes 1 and 2 and the X-chromosome indicated preferential breakage in the region of the centromere of each of these chromosomes, and in the long arms of chromosomes 1 and 2. On the assumption that hydroxylamine specifically affects the cytosine-guanine base pairs in the DNA molecule, it seemed reasonable to interpret these results as indicating a high cytosine-guanine content in those regions of the chromosomes exhibiting preferential breakage. On the basis of these studies, it was desirable to test the effects of hydroxylamine in a different biological system, namely plant meristem cells.

Treatment of cells with 5-fluorodeoxyuridine (5-FUDR) results in an inhibition of the activity of thymidylate synthetase, an enzyme responsible for catalyzing the conversion of deoxyuridylic acid to thymidylic acid. When the formation of thymidylic acid is prevented, DNA synthesis is inhibited. In effect, then, 5-FUDR serves as an inhibitor of DNA synthesis. Several workers have studied the effects of this agent on plant chromosomes, particularly in the roots of *Vicia faba*[7-10].

Considerable chromosome breakage results from treatment with 5-FUDR, and the frequency of X-ray-induced breaks is increased in the presence of this agent. Little or no reunion of broken ends of chromosomes was observed under the conditions employed by TAYLOR et al.[9], and the effect of 5-FUDR was considered to occur primarily during the period of DNA synthesis. In addition, these workers concluded that the synthesis of all four nucleotides of DNA is required for reunion, especially since the addition of thymidine and uridine resulted in a decrease in the inhibitory effect of the agent. On the other hand, KIHLMAN[7] showed that reunion does occur to some extent, since the frequency of exchanges induced by X-rays is not reduced in the presence of 5-FUDR, and a low level of reunion occurs with 5-FUDR treatment alone. One of the most striking effects of this agent is the production of shattered chromosomes. So many breaks may occur in a single cell that quantitative analysis is not possible (Figures 2–4). There is little doubt that the level of reunion (such as the production of exchanges) is considerably less than found with other agents causing breakage. Yet, some question remains concerning the cause of this inhibition of reunion. There is also conflicting evidence regarding the time of action of this agent in the production of chromosomal breaks. Although TAYLOR et al. indicated that the period of DNA synthesis is the time during which breaks are produced rather than during the post-synthetic period, the results of experiments in this laboratory are not wholly consistent with theirs.

A. *Studies with hydroxylamine*

Materials and Methods. Lateral roots of *Vicia faba* and bulb roots of *Allium cepa* were grown and treated in the dark at 23°C. Concentrations of hydroxylamine used ranged from $10^{-6} M$ to $10^{-2} M$, and treatment times were varied from 30 min to 24 h. In certain of the experiments, treatments were performed under different conditions of pH ranging from 4.45 to 10.35. Following treatment, roots were fixed at various times from 4 to 48 h in Carnoy's solution. When the recovery period exceeded 4 h, the roots were treated with 0.05% colchicine for 4 h prior to fixation. Permanent slides were prepared by the Feulgen method. Slides were made and cytological analyses performed in the experiments with hydroxylamine by Miss M. A. KNIGHT.

Observations. The results of experiments with *Allium cepa* are summarized in Table I. These results show that relatively few cells were affected by the treatment, but

1 E. FREESE, in *Molecular Genetics*, Part I (Ed. J. H. Taylor, 1963), p. 207.
2 R. FRANKLIN and E. WECKER, Nature *184*, 343 (1959).
3 E. FREESE, E. B. FREESE, and E. BAUTZ, J. molecular Biol. *3*, 133 (1961).
4 E. FREESE, E. BAUTZ, and E. B. FREESE, Proc. Nat. Acad. Sci., Wash. *47*, 845 (1961).
5 H. SCHUSTER, J. molecular Biol. *3*, 447 (1961).
6 C. SOMERS and T. C. HSU, Proc. Nat. Acad. Sci., Wash. *48*, 937 (1962).
7 B. A. KIHLMAN, Exp. Cell Res. *27*, 604 (1962).
8 B. A. KIHLMAN, Caryologia *15*, 261 (1962).
9 J. H. TAYLOR, W. F. HAUT, and J. TUNG, Proc. Nat. Acad. Sci., Wash. *48*, 190 (1962).
10 B. A. KIHLMAN, Hereditas *49*, 352 (1963).

those cells in which chromosome breaks occurred exhibited a relatively high frequency of breaks. This result is in general agreement with that found by SOMERS and HSU[6] in mammalian cells. At least one cell in each population of 100 metaphases analyzed for a given treatment showed shattering of the chromosomes, and in no case was there any evidence for reunion of broken ends of chromosomes. Consequently, no exchange aberrations were produced, contrary to the observations in Chinese hamster cells. Evidently, one of the effects of hydroxylamine is to reduce the amount of restitution or reunion. As will be discussed below in connection with the effects of 5-FUDR, it is quite possible that this *apparent* inhibitory effect of hydroxylamine is due to a disruption of DNA metabolism perhaps during the period of DNA synthesis.

A higher concentration of hydroxylamine, $5 \times 10^{-3} M$, was enough of an increase over $10^{-3} M$ to cause significant mitotic inhibition and cell death. This conclusion is based on observations made for treatment times of 1, 2, 4, and 24 h followed by fixations at 4 or 24 h in each case; in most instances, there was considerable lethality or relatively few divisions. In Table I, a difference can be noted between the effects of hydroxylamine at the two pH values utilized, an observation not readily explained in the light of previous observations in other organisms.

Results of various treatments of *Vicia faba* roots are shown in Table II. As was found in *Allium*, relatively few cells exhibited breaks in the chromosomes, and in no case was there any reunion of broken ends. Isochromatid deletions were all of the sister non-union type (NUpd), and no exchanges were observed. However, some exchanges were observed in cells treated with $10^{-3} M$ hydroxylamine for 24 h. The S/L ratios at the two lower pH values indicated, in addition, that there was localized breakage. (S/L indicates the ratio of the number of breaks in the short chromosomes to the number of breaks in the long chromosomes.) This observation was further substantiated by the fact that most of the breaks occurred in the heterochromatin of the long arms of the short chromosomes, in agreement with the observations in

Table I. Metaphase breaks resulting from treatment of *Allium cepa* roots for 4 h with $10^{-3} M$ hydroxylamine. (Fixations were made 24–25 h after the initiation of treatment)

pH	Frequency of normal cells[a]	Frequency of abnormal cells	Frequency of isochromatid deletions	Frequency of chromatid deletions
4.7	0.92	0.08	0.33	0.32
6.5	0.98	0.02	0.09	0.08

[a] Based on 100 cells (a minimum of 200 cells was analyzed for each treatment).

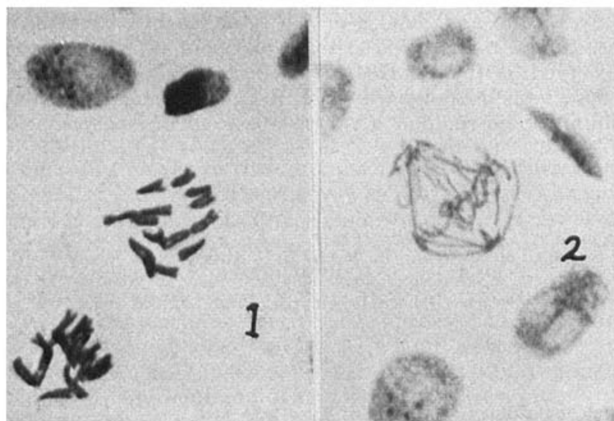

Fig. 1. Normal mitotic metaphase in *Vicia faba*.

Fig. 2. Anaphase cell from root of *Vicia faba* treated for 1 h with $10^{-5} M$ 5-FUDR and fixed at end of treatment.

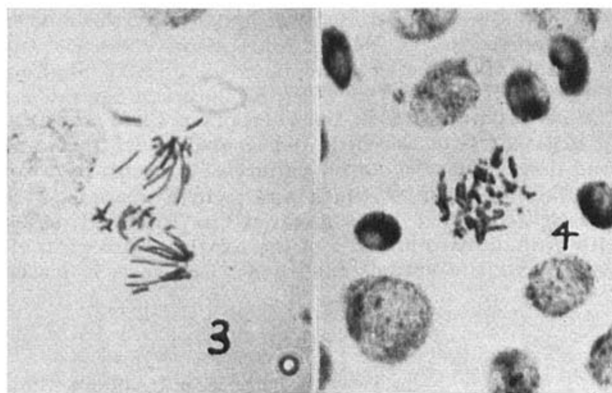

Fig. 3. Anaphase cell from root of *Vicia faba* treated for 5 h with $10^{-6} M$ 5-FUDR and fixed at end of treatment.

Fig. 4. Metaphase cell from root of *Vicia faba* treated for 1 h with $10^{-5} M$ 5-FUDR and fixed 3 h later.

Table II. Metaphase breaks resulting from treatment of *Vicia faba* roots for 4 h with $10^{-3} M$ hydroxylamine. (Fixations were made 24 h after end of treatment)

pH	Frequency of normal cells[a]	Frequency of abnormal cells	Frequency of isochromatid deletions	Frequency of chromatid deletions	S/L	Mitotic index
4.45	0.94	0.06	0.07	0.21	7 /1	0.09
6.75	0.92	0.08	0.05	0.33	3.5/1	0.07
10.35	0.96	0.04	0.09	0.11	2 /1	0.14
7.00 (Control, H_2O)	1.00	0.00	0.00	0.00	–	0.15

[a] Based on 100 cells (a minimum of 200 cells was analyzed for each treatment).

Chinese hamster cells. At a pH of 10.35, the S/L value was indicative of a random breakage pattern rather than preferential breakage. It is possible that at this pH hydroxylamine is less specifically reactive in the heterochromatic regions of the short chromosomes. This result is consistent with the studies of FREESE et al.[4] in which the reactivity of cytosine with hydroxylamine was 30 times greater at pH 6.5 than at pH 9.5.

Vicia roots treated under the three conditions of pH (Table II) with $10^{-6}M$ or $10^{-2}M$ hydroxylamine and fixed 4 or 8 h after treatment exhibited no significant cytological effect other than apparent mitotic inhibition. On the other hand, cells treated for 2 h with $10^{-2}M$ hydroxylamine and fixed 44 h after the end of treatment exhibited a significant frequency of anaphase bridges. This suggests that reunions did occur earlier. Additionally, contrary to the results of SOMERS and HSU[6], no multiple constrictions were found in the chromosomes with any treatment, and much higher concentrations of hydroxylamine were required to produce cytological effects in *Vicia* as compared with Chinese hamster cells.

At least two interpretations of the effect of hydroxylamine in producing chromatid breaks are possible. Either the cytosine-guanine base pairs are directly affected by the agent, as suggested by preferential breakage patterns, or the base neighbors of cytosine-guanine in these regions of the short chromosomes are more susceptible to breakage by hydroxylamine than are cytosine neighbors in the long chromosomes or in other (euchromatic) regions of the short chromosomes. As suggested above, the effect of hydroxylamine on the rejoining processes and on mitosis may be based upon some interference with DNA metabolism, although the several results indicate that breakage may be induced during or after the period of DNA synthesis.

B. *Studies with 5-fluorodeoxyuridine*

Materials and Methods. Lateral roots of *Vicia faba* were grown at 21°C in the dark and treated under the same conditions. The effects of a variety of concentrations of 5-FUDR[11], treatment times, and recovery periods were investigated. These are summarized in Table III, along with a qualitative analysis of the relative cytological effects under the various conditions. Emphasis is placed primarily upon specific aspects of the experimental results: whether or not breakage, shattering of chromosomes, and reunion of broken ends occurred. Quantitative data, although obtained in these investigations, are of little real value in terms of analyzing the effects of this particular agent under the conditions employed.

Observations. The low level effect of $10^{-8}M$ is in agreement with the results of KIHLMAN, who observed no effect at a concentration of $10^{-7}M$ for a 3-h treatment. At a concentration of $10^{-5}M$ the effect was substantial (Figures 2 and 4), but it is quite possible that all of the damaged cells were not recovered due to mitotic inhibition and lethality. With this concentration of 5-FUDR an effect was evident as early as 3 h after a 30-min treatment, or 1 h following a 1- or 2-h treatment (Figure 2). Since these cells were studied during metaphase or anaphase, it is extremely unlikely that they were engaged in DNA synthesis at the time of treatment.

The cell cycle in *Vicia faba* has been well substantiated[12], and is known to exhibit approximately the following sequence of events: period of DNA synthesis, 6 h; post-synthetic period, 4–6 h; division cycle (mitosis), 1–4 h. Thus, cells fixed within 3 or 4 h following treatment and showing chromosome breakage at metaphase or anaphase could not have been in the synthetic period at the time of treatment.

More extensive studies were performed using a concentration of $10^{-6}M$, since this concentration was used by other workers[8,9], and at this concentration the effect is significantly high but low enough to permit recovery of a substantial number of mitotic figures for analysis (Figure 3). Chromatid breaks were observed in cells treated for only 15 min and fixed 3 or 8 h later. If cells were fixed less than 3 h after this 15-min treatment no breaks were observed. With a 1-h treatment and fixation 3 or 4 h later, appreciable breakage was observed at metaphase and anaphase. Some reunion was also observed in isochromatid

[11] Courtesy of Hoffmann-La Roche Inc., Nutley, New Jersey.

[12] J. READ, *Radiation Biology of Vicia Faba in Relation to the General Problem* (Blackwell Scientific Publ., Oxford 1959).

Table III. Summary of treatments with 5-fluorodeoxyuridine in the roots of *Vicia faba*

Concentration	Treatment times	Recovery periods	Results
$10^{-5}M$	30 min 1 h 2 h	1, 3, 6, 8, or 24 h	little reunion; some shattering of chromosomes, even with 30 min treatment; a significant level of breakage for all treatments except in cells fixed at 24 h
$10^{-6}M$	15 min 30 min 1 h 2 h 3 h 4 h 5 h 6 h 7 h 8 h	immediate fixation and 12, 24, and 50 h recovery periods for each	some breakage with 15 min treatment in cells fixed 3 or 8 h later – no shattering; cells treated for 1 h and fixed 3 or 4 h later show metaphase and anaphase breaks; anaphase bridges indicate some reunion; 2, 3, 4, 5, and 6 h treatments and immediate fixation yielded much breakage, some reunion; several cells with shattered chromosomes
$10^{-8}M$	15 min 30 min 1 h 2 h	immediate fixation, and 12 and 24 h recovery periods for each	very little breakage, no mitotic inhibition

breaks, and exchanges were found at metaphase and bridges at anaphase. The presence of the latter types of aberrations indicates a prior reunion of broken chromosome ends. Treatment for 2, 3, or 4 h with immediate fixation at the end of the treatment time yielded breakage and some reunion. These results provide significant support for a post-synthetic effect of 5-FUDR.

Cells treated for 1 h or more and fixed 12 h (but not as late as 24 h) exhibited numerous breaks, but less reunion of broken ends than was found in cells fixed earlier than 12 h. Thus, breaks can be induced both during and following the period of DNA synthesis, and less reunion occurs during the period of synthesis than during post-synthetic periods. In addition, the earlier the time of treatment during the cell cycle, the greater is the level of mitotic inhibition. Roots treated for 1, 2, or 3 h and fixed immediately thereafter had almost as many mitotic figures as found in the control slides (Figure 1). Most other treatments resulted in severe mitotic inhibition. Thus, the inhibition of mitosis also appears to be greatest when the cells are treated during or close to the period of DNA synthesis. In conclusion, evidence has been obtained to support the contention that 5-FUDR produces cytological damage not only during the synthetic period in the cell cycle, but also during the post-synthetic period and during early stages of mitosis. At the same time, reunion of broken ends of chromosomes is greatly reduced, especially when cells are exposed during DNA synthesis or close to that period.

Conclusions. Several interesting comparisons between the effects of 5-FUDR and hydroxylamine are possible. From the standpoint of radiomimesis, both 5-FUDR and hydroxylamine produce the kinds of cytological damage to be expected. Of particular interest is the fact that their specific effects in the cell are based upon quite different chemical activities. Hydroxylamine appears to affect the base composition of DNA, while 5-FUDR results in the inhibition of DNA synthesis. Yet both agents are capable of causing breaks in the chromosomes near or during the period of DNA synthesis as well as during post-synthetic periods. However, hydroxylamine produces drastic effects in a few cells, whereas 5-FUDR affects a larger cell population. The apparent inhibition of rejoining (restitution and reunion) by either agent is difficult to explain. There is evidence that nucleotide synthesis is required for reunion of breaks induced by 5-FUDR[9], as might be expected when the breaks occur during the synthetic period. However, the rejoining of breaks induced at a later time may not be related to DNA synthesis. Rejoining of breaks induced during late interphase or prophase may be somewhat impaired because of chromosome movements, but it seems unlikely that this alone can account for the considerable absence of reunion or restitution. Since many of the treatments involved rather long exposures to the agent used – 1 h or longer – one might expect that the breaks were induced throughout this treatment period such that relatively few were available for reunion at any one time. However, studies with other radiomimetic agents do not support such a contention. In addition, the implication that these two particular agents produce more extensive damage in the chromosomes because of their specific chemical effects on the structural components of the chromosomes (presumably mainly DNA), may explain the lack of rejoining.

Among the more significant conclusions to be drawn from these studies are: (1) 5-FUDR treatments suggest the importance of DNA metabolism in the phenomena of restitution and reunion, (2) hydroxylamine is a radiomimetic agent that apparently has as its basis of effect specific influence on the cytosine-guanine pairs of the DNA molecule. This latter conclusion has important implications in terms of the organization of the chromosome, since the majority of damage appears to have occurred in the heterochromatic regions of the chromosomes. Finally, it should be indicated that DNA is not the only structural component of the chromosomes, but that histone protein is also quite important. Thus, chromosome breakage must certainly involve disruption of protein along with DNA. Current investigations are concerned with this aspect of radiomimesis[13].

Résumé. L'hydroxylamine et la 5-fluorodeoxyuridine provoquent des altérations notables dans les chromosomes de méristèmes radiculaires (*Vicia* et *Allium*). Ces deux agents empêchent aussi, dans une large mesure la réunion des segments après ruptures des chromosomes. Il est probable que l'hydroxylamine affecte spécifiquement les régions hétérochromatiques.

N. S. Cohn

Department of Botany, Ohio University, Athens (Ohio, U.S.A.), June 28, 1963.

[13] This research was supported by the U.S. Atomic Energy Commission under Contract AT(11-1) 826. – The author expresses his appreciation of those phases of the work contributed by Miss M. A. Knight whose research was part of an Honors Project at Ohio University.

γ-Aminobutyric Acid and Crab Muscle Fibres

The action of γ-aminobutyric acid (GABA) on crustacean muscle fibres has been investigated by several groups of authors[1–6]. In the case of crayfish[1] and lobster[2] muscle fibres, the application of GABA produces a marked increase in membrane conductance as does the transmitter substance of the inhibitor neurons. For crab muscles several authors[3–5] have reported no effect of GABA on membrane conductance although the substance blocks the excitatory junction potentials. However, in a recent note[6] it was demonstrated that GABA causes an increase in membrane conductance in fibres of the opening and closing muscles of *Cancer borealis*. This result is in conflict with that obtained by other workers in the closing muscle of the same species[5].

[1] J. Boistel and P. Fatt, J. Physiol. *144*, 176 (1958).
[2] H. Grundfest, J. P. Reuben, and W. H. Rickles, J. gen. Physiol. *42*, 1301 (1959).
[3] G. Hoyle and C. A. G. Wiersma, J. Physiol. *143*, 426 (1958).
[4] E. Florey and G. Hoyle, in *Nervous Inhibition* (Edit. by E. Florey, Pergamon Press, 1961).
[5] E. Aljure, H. Gainer, and H. Grundfest, Biol. Bull. *123*, 479 (1962).
[6] R. S. Eisenberg and D. Hamilton, Nature *198*, 1002 (1963).