An. Aula Dei (1988), 19 (1-2): 89-114.

Mechanism of G2-Repair to preserve chromosome integrity and its inhibition by caffeine*

by **J.F. LOPEZ-SAEZ[1], A. GONZALEZ-FERNANDEZ, P. HERNANDEZ, E. ZAMORANO[2] y M.H. NAVARRETE**

Centro de Investigaciones Biológicas, CSIC.
Velázquez, 144. 28006-Madrid. España.
1) Departamento de Biología. Facultad de Ciencias.
Universidad Autónoma de Madrid. 28049-Madrid
2) Instituto Profesional. Chillán. Chile.

Key Words: chromosomal aberrations, G2-prophase repair, DNA repair, caffeine.

ABSTRACT

J.F. López-Saez, A. González-Fernández, P. Hernández, E. Zamorano y M.H. Navarrete. 1988. Mechanism of G2-Repair to preserve chromosome integrity and its inhibition by caffeine. *An. Aula Dei* 19 (1-2): 89-114.

In proliferating eukaryotic cells, there occurs a striking chromosome repair mechanism, which operates during G2 and prophase of the division cycle. The main task of this repair mechanism could by the removal of DNA lesions able to give rise chromosomal aberrations during chromatin condensation and chromosome movements in next mitosis. In the present work, we study this repair mechanism, its inhibition by caffeine and we propose a model to explain the estrategy of cell mechanism operating under control conditions and after the treatment with any clastogenic agent.

The G2-prophase repair is a mechanism of eukaryotic cells reminiscent of bacterial SOS system and it would operate as follows: I) The response is induced by the presence of certain DNA lesions in post-replicative and premitotic genome, whenever they were induced. Under these circumstances, a cell cycle arrest takes place in G2, also termed division delay. II) During the G2 arrest, DNA repair pathways operate to restore DNA structure. III) When adequate DNA structure for physical chromosome integrity is achieved cells go into mitosis. This recovery from arrest is dependent on protein synthesis.

Caffeine is an inhibitor of G2-prophase repair mechanism by cancelling division delay and, probably, by affecting the rate of DNA repair at the same time. Caffeine efficiency appears to by dependent on drug concentration and cellular ATP level. The lower the level the higher its inhibitory efficiency.

Cycloheximide can ameliorate DNA repair during G2-prophase, even in the presence of caffeine. Likely, inhibition of protein synthesis during post-treatments with damaging agents allows damaged cells to have enough time for DNA repair before mitosis.

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* A nuestro amigo Enrique, que tanto entiende de la belleza e integridad del cromosoma.

INTRODUCTION

A large body of literature has grown up around the potentiating effect of caffeine on chemically or physically induced chromosome aberrations, extensively reviewed by Kihlman (1977), Roberts (1978) and Dews (1984).

The first evidence reported on this topic was published in 1969 by Yamamoto and Yamaguchi using *Hordeum vulgare* as experimental material. They demonstrated that the frequency of chromatid aberrations induced by γ-irradiation was increased when meristematic cells were treated with caffeine during the G2 phase.

Along the seventies there were many reports on caffeine enhancement of radiation or chemically induced chromosome damage in plants (Schoneich et al, 1970; Swietlinska, 1971; Switlinska et al, 1973; Kihlman et al, 1973; Kihlman and Sturelid, 1974) and animal cells (Hartley-Asp and Kilman, 1971; Shalumashvili, 1972; Waldren, 1972; Brogger, 1974; Frei and Venitt, 1975; Nilsson and Lehman, 1975; Roberts et al, 1977 and 1978).

All those findings strongly suggest that the exposure of proliferating cells to chromosome damaging agents produces lesions which repairing takes place during the S period or the next G2-prophase (Kihlman and Andersson, 1980; Murnane et al, 1980; Palitti et al, 1983).

In onion root meristem cells, post-treatments with caffeine during the G2 phase dramatically enhance the yield of 5-aminouracil-induced chromosome damage (González-Fernández and López-Sáez, 1982). In the same material, it has been also demonstrated that the treatment with this agent during the late S period delays mitosis. Post-treatment with adenosine alone has shown no effect on mitotic delay or chromosome damage. However, when cells were postreated with caffeine plus adenosine the chromosome damage potentiation was partially abolished without affecting the caffeine cancelation of mitotic delay (González-Fernández et al, 1985). Recently, it has been claimed that the ATP level and/or something correlating with it could explain the efficiency of caffeine in inducing chromosomal aberrations. In short, the lower the ATP level the higher the caffeine efficiency (Hernández et al, 1986).

In the present paper we have studied, reviewed and discussed results obtained by us in *Allium cepa* and a substantial part of the paper has been devoted to analyse evidences from other materials in order to understand something about the chromosome repair phenomena which take place during G2 and prophase of the cell division cycle.

MATERIAL AND METHODS

The material used was *Allium cepa L.* root meristems. They were grown in tap water in the dark at a constant temperature (± 0.5 °C), indicated in each experiment. The capacity of the cylindrical glass receptacles was approximately 80 ml. The tap water was renewed at 24-h intervals, and was aerated by continuous bubbling at the rate of 10-20 ml air/min. The bulbs were positioned such that only their bases remained submerged in the water.

Treatment

The culture conditions just described were maintained throughout the period of treatment and the solution concentrations were as follows. 5-Aminouracil (Sigma) was used at a concentration of 0.5 mM. Caffeine (Merck) was used at the various concentrations specified for each experiment.

Labelling with [^{3}H] thymidine

[Me-^{3}H] Thymidine (^{3}HdThd from the Radiochemical Centre, Amersham, Great Britain) with a specific activity of 925 GBq/mmole was used at a concentration of 370 KBq/ml. The autoradiography was carried out with Kodak stripping film AR-10 which was developed, after 3 weeks' exposure, in a Kodak D-19 developer and fixed with a Kodak ultrarapid acid fixer.

Cytological procedures

For cytological analysis the roots were fixed in a 3:1 ethanol-acetic acid mixture, and the specimens were prepared by staining the roots with acetic orcein according to the Tjio and Levan technique (1950). The mitotic index was determined by the method used by López-Sáez and Fernández-Gómez (1965).

We use the term "abnormal ana-telophases" to describe those cells in anaphase or telophase that showed any chromosomal aberrations (bridges, fragments, etc.). We determined the percentage of abnormal ana-telophases compared to the total ana-telophases scoring 4 meristems, about 1000 ana-telophases for each point.

Extraction and assay of adenine nucleotides

In each assay, 10 roots were placed over a slide previously frozen with dry ice. After the roots were frozen, the first 0.7 mm from each root apex was cut off and discarded, the next 2 mm being taken. These meristems, corresponding to the sample under study, were homogenized with 0.1 ml of cold 60% $HClO_4$ and 0.5 mol of cold distilled water. The homogenate was transferred to a test tube and maintained at -10 °C for 30 min with frequent vigorous shaking. The resulting suspension was frozen with dry ice and then allowed to thaw. This process was repeated twice to liberate quantitatively the cell metabolites. The final suspension was centrifuged in the cold for 10 min at 10.000 x g and the precipitate used for protein determination by the method of Lowry et al. (1951). The supernatant was adjusted to pH 6.5 with a solution containing 1.8 mM KOH and 1.6 mM $HKCO_3$. After 15 min at 0 °C, the precipitate was centrifuged down. The supernatant was utilized for the fluorometric determination of adenine nucleotides by the method previously described by Williamson and Corkey (1968).

RESULTS AND DISCUSSION

Potentiation of clastogenic action of 5-AU by caffeine

The meristem cells of onion roots growing under steady-state conditions suffer only very little chromosome damage and in these circumstances it would be difficult to estimate with accuracy any anti-repair effect. Therefore, we decided to use a pretreatment with 5-AU to induce both chromosome damage and mitotic synchronization (Navarrete et al, 1984).

When roots were exposed to 0.5 mM AU for 18h, during the recovery in tap water we observed a mitotic wave as shown in Fig. 1, the mitotic peak appears 10 h later and the highest frequency of ana-telophases was reached 2 h after this time, i.e. 12 h after the end of AU treatment. The chromosome damage induced by AU was estimated as the frequency of ana-telophases with chromosomal aberrations at this time.

The results of caffeine incubations for 2 h during the recovery are summarized in Fig. 2 and the harvesting time was always 12 h after the removal of AU. The frequency of abnormal ana-

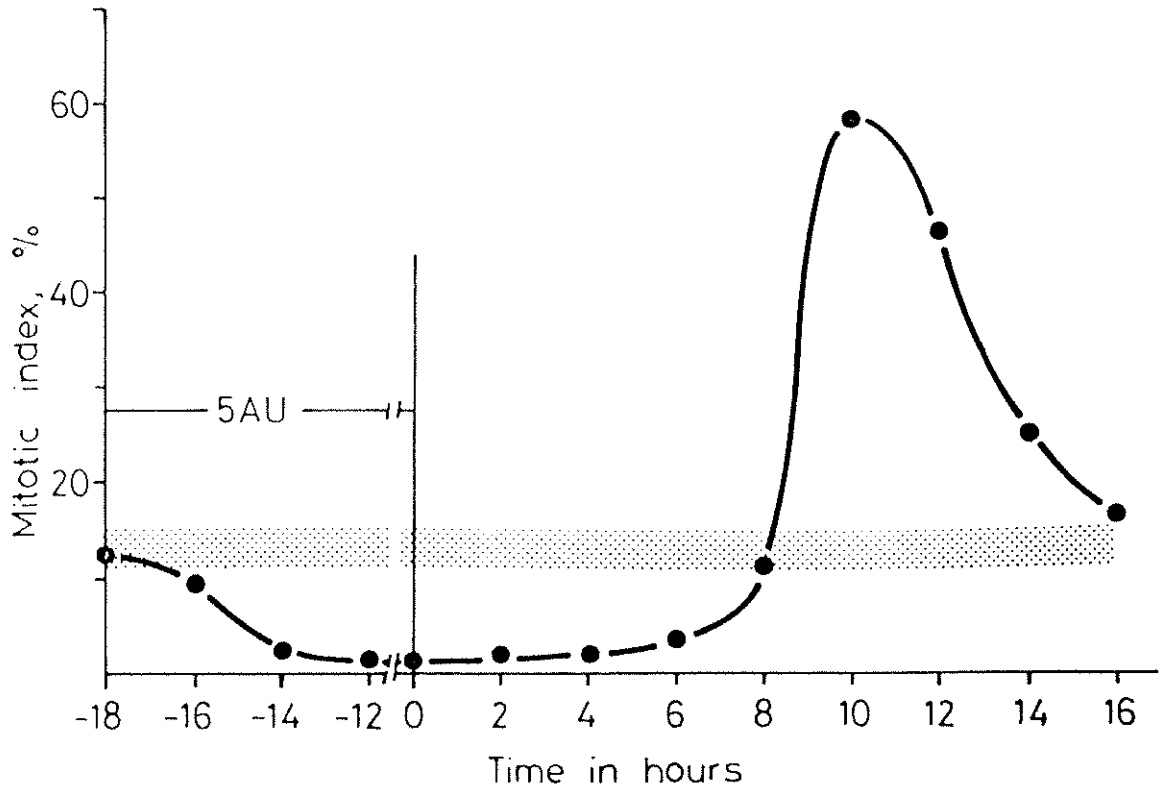

Fig. 1.- Mitotic synchrony wave induced by an 18-h treatment with AU (0.5 mM). The dotted band represents the normal mitotic index.

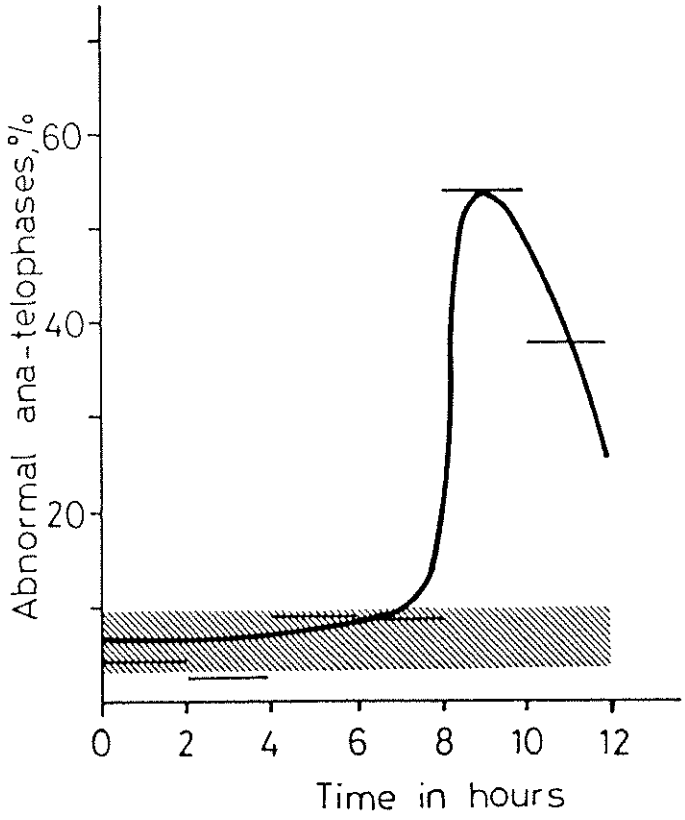

Fig. 2.- During the recovery after an 18-h treatment with 0.5 mM AU, the roots were post-treated with 2-h pulses of 5 mM caffeine at various times. Post-treatment from the 8th to the 10th h strikingly enhanced the percentage of abnormal ana-telophases induced for AU alone. The stripped band represents the frequency of abnormal ana-telophases induced by AU. The harvesting time was always 12 h after the removal of AU.

telophases obtained when AU-treated cells were caffeine post-treated from the 8th h to the 10th h were about 7 times higher than those produced by the synchronizing agent alone, and about 4 times higher than the level induced by 5mM caffeine on control roots.

a) *Caffeine-sensitive region*

Because the maximal potentiating effect of caffeine was detected when were post-treated from 4 to 2 h before harvesting, it was logical to assume that this sensitive region was located in the G2 period. In fact, the incubation during this time with tritiated thymidine demonstrated that it was autoradiographically out of the replicative period (Table I). Moreover, the strong potentiating effect induced by caffeine when incubation begun 2 h before fixation suggested that the sensitive region included at least part of prophase.

Thus, the *caffeine-sensitive region* coincides with the *G2-prophase period* of the cell cycle.

b) *Reversion of the caffeine effect by adenosine*

During the last years, our group has suggested (González-Fernández and López-Sáez, 1980; López-Sáez et al., 1982) that the effect of caffeine on cytokinesis, calcium transport, DNA repair, phosphodiesterase activity, and purine-metabolizing enzymes could be explained by a weak affinity this drug might have to either substrate sites or to allosteric effector sites for adenine nucleotides of some enzyme(s) involved in each process.

To test the reversion of the caffeine-potentiating effect on AU-induced chromosomal aberrations, we selected 0.5 mM adenosine as the concentration that induces the best reversion of caffeine cytokinesis inhibition and the highest increase in ATP level under similar conditions. Table 2 shows that 0.5 mM adenosine not only halved the effect of 5 mM caffeine but also that of 7 mM caffeine.

c) *Damage induced by AU in short incubations*

When root meristems are incubated in 0.5 mM AU for 3 h, the mitotic index drops from a control value about 12% to low values (about 1%) during recovery and a fairly constant level of abnormal ana-telophases (15-20%) appears during some hours.

Under similar conditions, a 2 h post-treatment with 3 mM caffeine increases the frequency of aberrations (Fig. 3) and reduces the fall in mitotic index (Fig. 4).

The experimental procedure designed to estimate the effect of caffeine and adenosine on G2 delay is illustrated by Fig. 5. In every case, a pulse with tritiated thymidine allowed estimation of the mean duration of the G2 period by scoring the frequency of labelled prophases in autoradiographs. Besides, the level of aberrations was always studied 5 h after the radioactive pulse (Table 3).

Under control conditions, the G2 period takes 2.8 h and the AU treatment induces an important G2 arrest in cells located in the late replication phase. 3 mM Caffeine reduces by 55% the AU-induced division delay and triples the frequency of abnormal ana-telophases (Fig. 6, Table 3).

Apparently, a post-treatment with adenosine alone does not affect the level of AU-induced chromosome damage and only marginally reduces the G2 delay, as was claimed by Tomasovic and Dewey (1978). However, a post-treatment with caffeine plus adenosine reduces G2 delay without enhancement of chromosomal aberrations (Table 3).

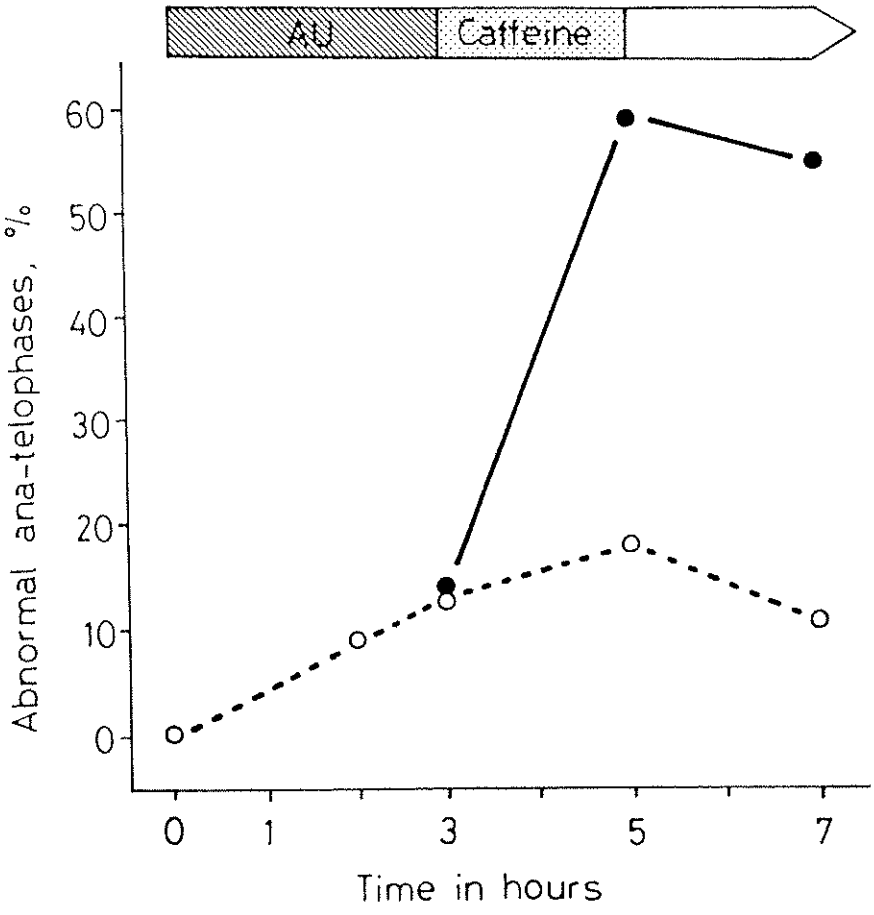

Fig. 3.- Percentage of abnormal ana-telophases observed at different times after a single 3-h treatment with 0.5 mM AU (O) or after such treatment followed by a 2-h treatment with 3 mM caffeine (●). Caffeine post-treatment clearly increases the frequency of aberrations.

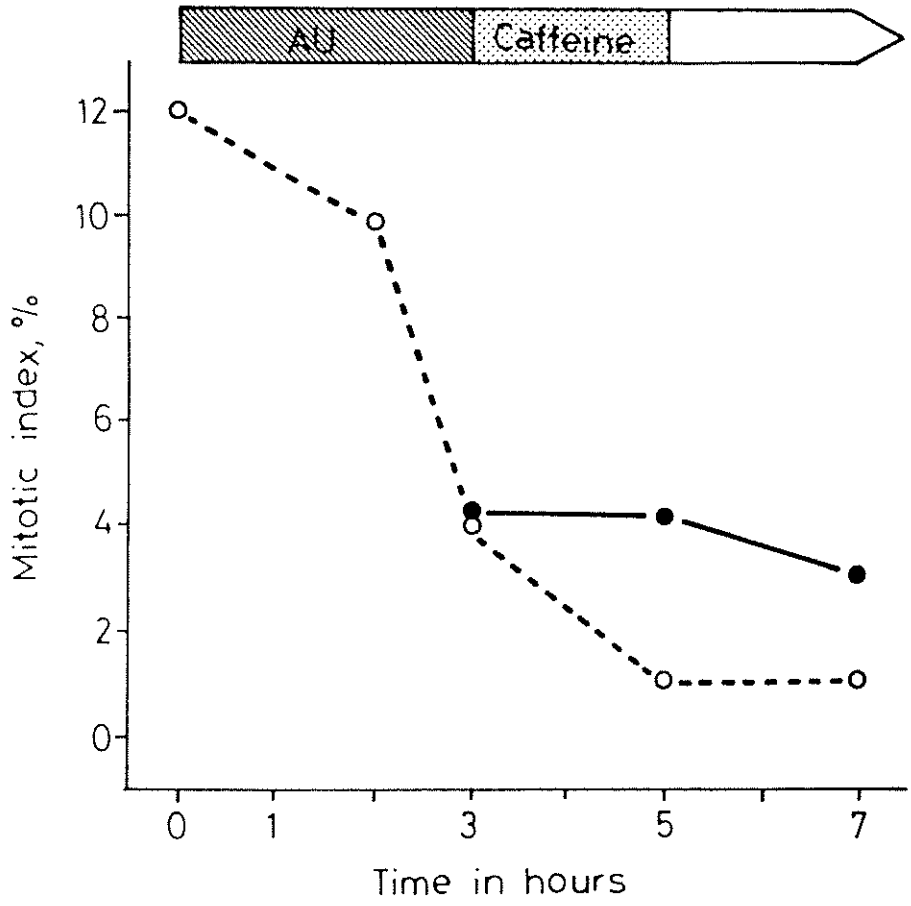

Fig. 4.- Mitotic index observed after a single 3-h treatment with o.5 mM AU (O) and after AU followed by a 2-h treatment with 3 mM caffeine (●). Caffeine post-treatment reduces the fall in mitotic index.

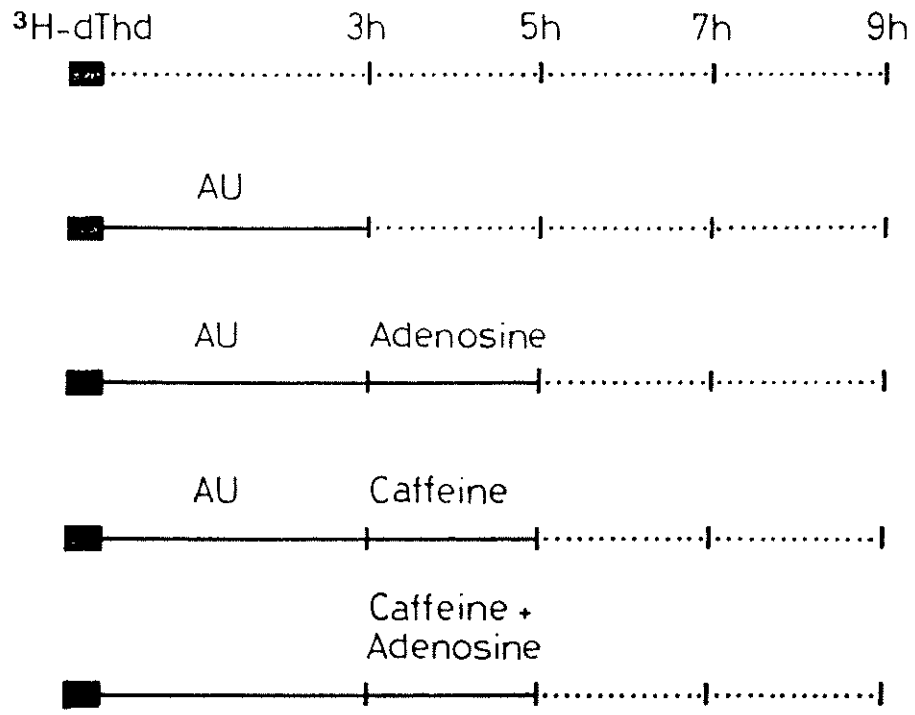

Fig. 5.- Experimental scheme designed to estimate the effect of caffeine and/or adenosine in G2 delay induced by a 3-h treatment with 5-aminouracil (0.5 mM AU, 3 mM caffeine, 0.5 mM adenosine).

Table 1.- Ana-telophases scored at the 12th h, after incubation in AU treatment from the 8th to the 10th h with tritiated thymidine alone or in presence of caffeine.

	Unlabelled	%	Labelled	%
^{3}H dThd	1082	99.63	4	0.37
^{3}H dThd + 5 mM caffeine	783	99.49	4	0.51

Table 2.- Percentage of abnormal ana-telophases scored at the 12th h after the 18-h AU treatment followed by 2-h treatment (from the 8th to the 10th) with either caffeine alone or caffeine plus adenosine.

	5 mM caffeine	5 mM caffeine + 0.5 mM adenosine	7 mM caffeine	7 mM caffeine + 0.5 mM adenosine
Abnormal ana-telophases (%)	53.2	19.3	59.7	28.2

Table 3.- Estimate of chromosome damage and G_2 duration after a tritiated thymidine pulse followed by different treatments

Treatment (3 h)	Post-treatment (2 h)	Abnormal[a] ana-telophases (%)	Duration of G_2 (h)	Mitotic delay (h)
–	–	0.5	2.8	–
0.5 mM AU	–	18.2	6.3	3.5
0.5 mM AU	0.5 mM adenosine	22.5	6.0	3.2
0.5 mM AU	3 mM caffeine	59.7	4.4	1.6
0.5 mM AU	Caffeine + adenosine	17.8	4.2	1.4

[a]The abnormal ana-telophases were analysed 5 h after addition of ^{3}H-dThd.

Table 4.- Adenine nucleotide level and proliferative rate at diferent growth temperatures (°C)

Growth temperature	Proliferation rate ($h^{-1}.10^3$)	ATP	ADP	AMP
		pmoles/μg protein		
5	7.7	13.2	2.8	2.5
10	18.3	15.0	2.6	2.4
15	33.5	20.5	3.3	2.2
20	53.2	29.6	1.4	1.2
25	74.0	39.0	0.8	1.1

Chromosomal damaged induced by caffeine alone during G2

The meristem cells growing under steady-state conditions suffer only little chromosome damage and the level of aberrations is negligible, indeed. Recently, it has been postulated (De Marco and Cozzi, 1980; De Marco and Polani, 1981; Kihlman et al., 1982b; González-Fernández and López-Sáez, 1982) that during late interphase chromatin condensation could lead to the induction of chromosome damage from preexistent lesions which would be readily repaired by a G2-prophase repair mehanism. In the presence of caffeine, this activity is blocked and the basal level of DNA lesions after one replication round would lead to the formation of chromosome aberrations. Fig. 7 summarizes the results obtained when roots were incubated at different caffeine concentrations for 2 h, at 25 °C. The kinetics of the response are very similar in every case, with a summit of aberrations close to the end of the treatment. Besides, the response was clearly dose-dependent and the caffeine-sensitive region appeared to be located in the G2-prophase period of the cell cycle.

a) *Growth temperature*

Since the caffeine-sensitive region coincides with the G2-prophase period of the cell cycle, as has been previously established, and the duration of this period is dependent on growth temperature, we have incubated the cells for time periods proportional to the duration of G2-prophase at each growth temperature (González-Fernández et al 1971). In order to cover equivalent segments of the caffeine-sensitive region, the roots were treated for 1 h at 25 °C, 2h at 15 °C, 4 h at 10 °C and 8h at 5 °C. In contrast with the previous results, the highest level of abnormal ana-telophases was obtained at 5 °C, and decreased with increasing temperature (Fig. 8).

In another experiment, a dose-response study of caffeine efficiency was carried out at 15 °C, 20 °C and 25 °C. The duration of treatments was again proportional to cell cycle time at each temperature, 4h at 15 °C, 3h at 20 °C and 2h at 25 °C. Fig. 9 shows the results obtained, where the lower the temperature, the greater the slope of the correlation.

b) *Caffeine efficiency and ATP level*

Recently, we have demonstrated that control meristems contain higher ATP level at higher growth temperature (Table 4) and that caffeine modifies the baseline ATP level in a concentration-dependent way (Table 5).

Moreover, by plotting the induced chromosomal damage observed at the end of the treatment against [caffeine]. $[ATP]^{-2}$, we have obtained a good linear correlation (Fig. 11, $r = 0.977$). The ATP value introduced in each case corresponds to the average of the values estimated before (control cells) and after caffeine incubation. It seems worthwhile to point out that the substitution of [caffeine]. $[ATP]^{-2}$ for caffeine, as shown in Fig. 10, cancels the effect of temperature in such a way that the three linear correlations (one for each temperature) become only one.

Therefore, it appears that the ATP level and/or something correlating with it not only is important, but also can explain the influence of growth temperature on caffeine efficiency in inducing chromosomal aberrations.

Potentiation of clastogenic action of MMS by caffeine

Methyl-methane sulfonate (MMS) has been an alkylating agent selected by our group to test its genotoxic action on cells located at late S period and the possible potentiation of this effect by caffeine.

Table 8 and Fig. 11 summarize the present results, which are very similar to that obtained

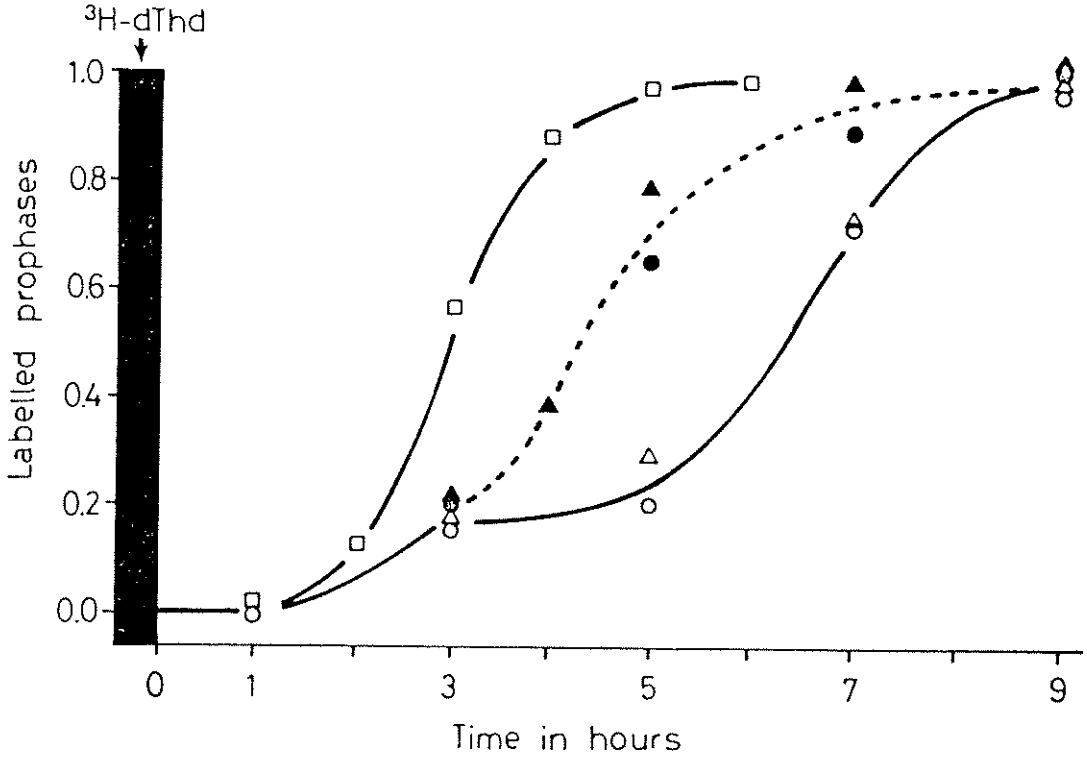

Fig. 6.- Frequency of labelled prophases after a 20-min pulse with ^{3}H thymidine. Under control conditions (□); after a 3–h treatment with AU(○) and AU followed by either a 2-h post-treatment with adenosine (△) or 2-h caffeine (●). Solid triangles (▲) represent the post-treatment with caffeine plus adenosine. Caffeine post-treatments reduce mitotic delay (see Table 3).

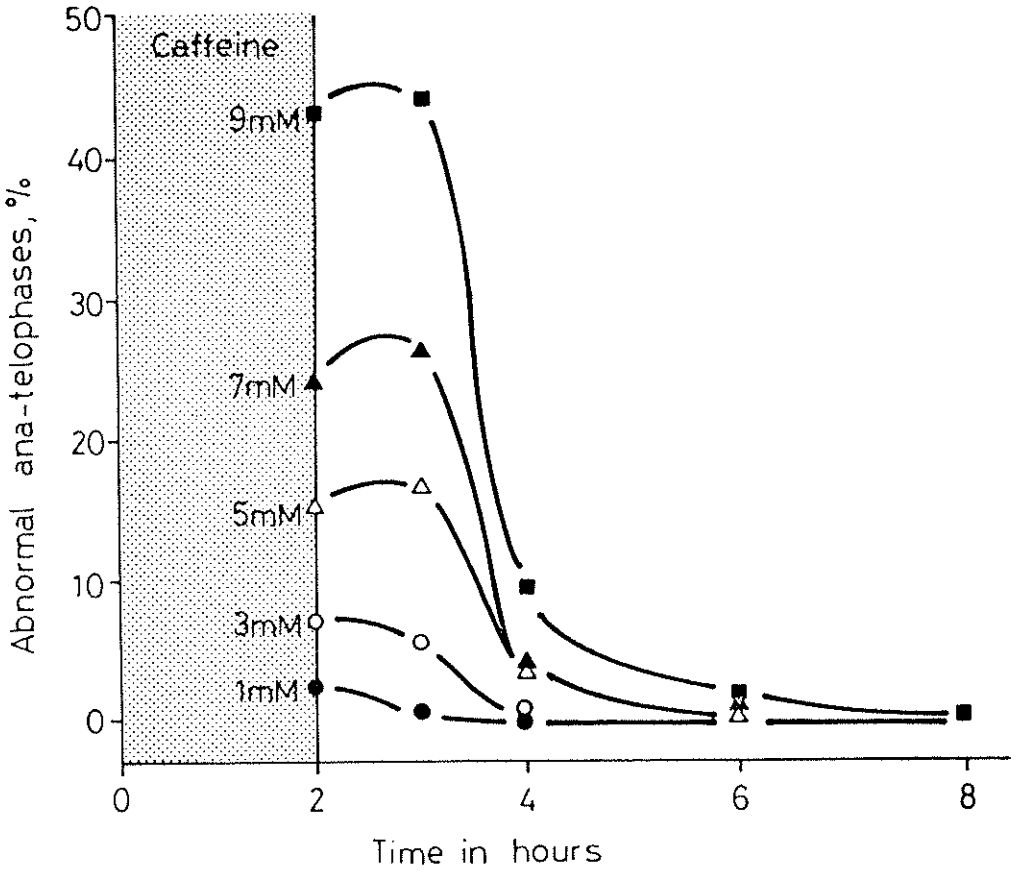

Fig. 7.- Percentage of abnormal ana-telophases observed at different times after 2-h treatments with caffeine at different concentrations, at 25 °C.

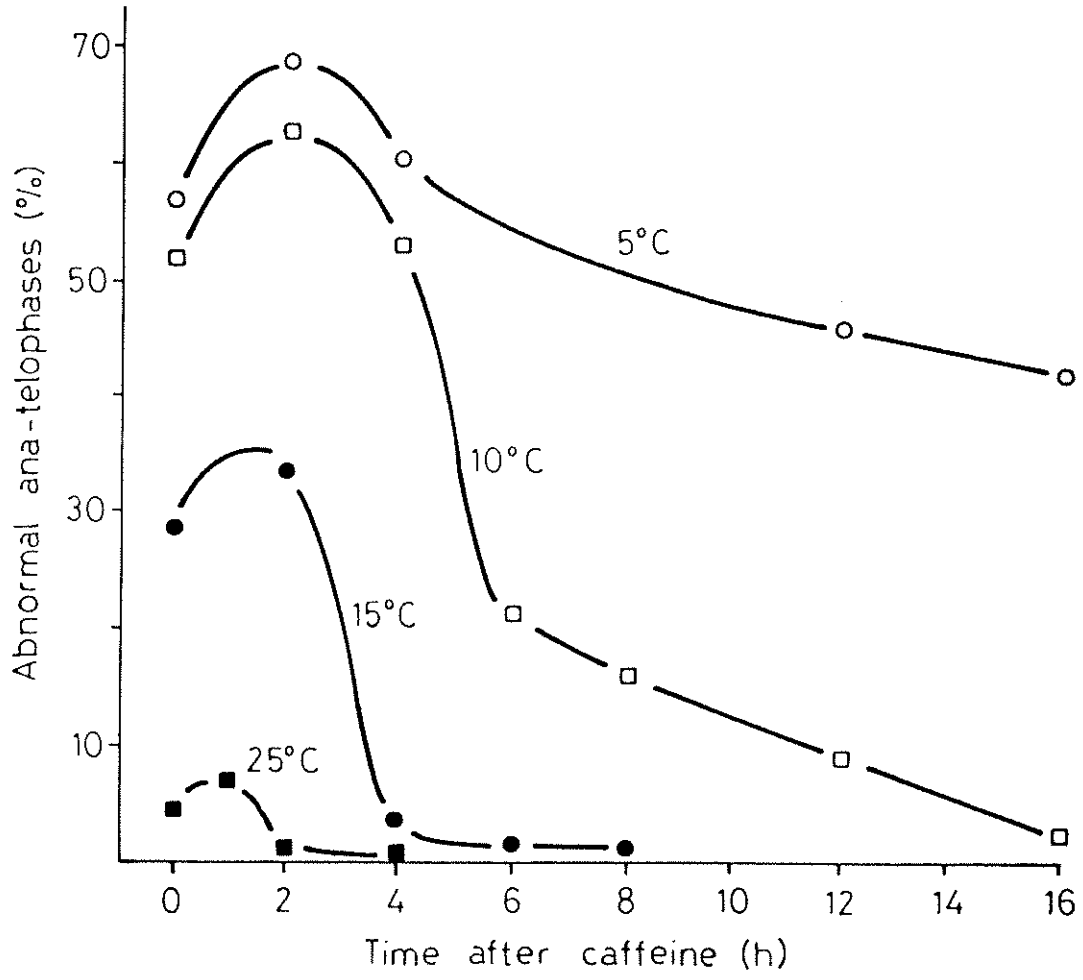

Fig. 8.- Percentage of abnormal ana-telophases observed at different times after 5 mM caffeine treatment under different growth temperature. Duration of treatment was proportional to the cell-cycle time at each temperature (1h at 25 °C, 2h at 15 °C, 4 h at 10 °C and 8 h at 5 °C).

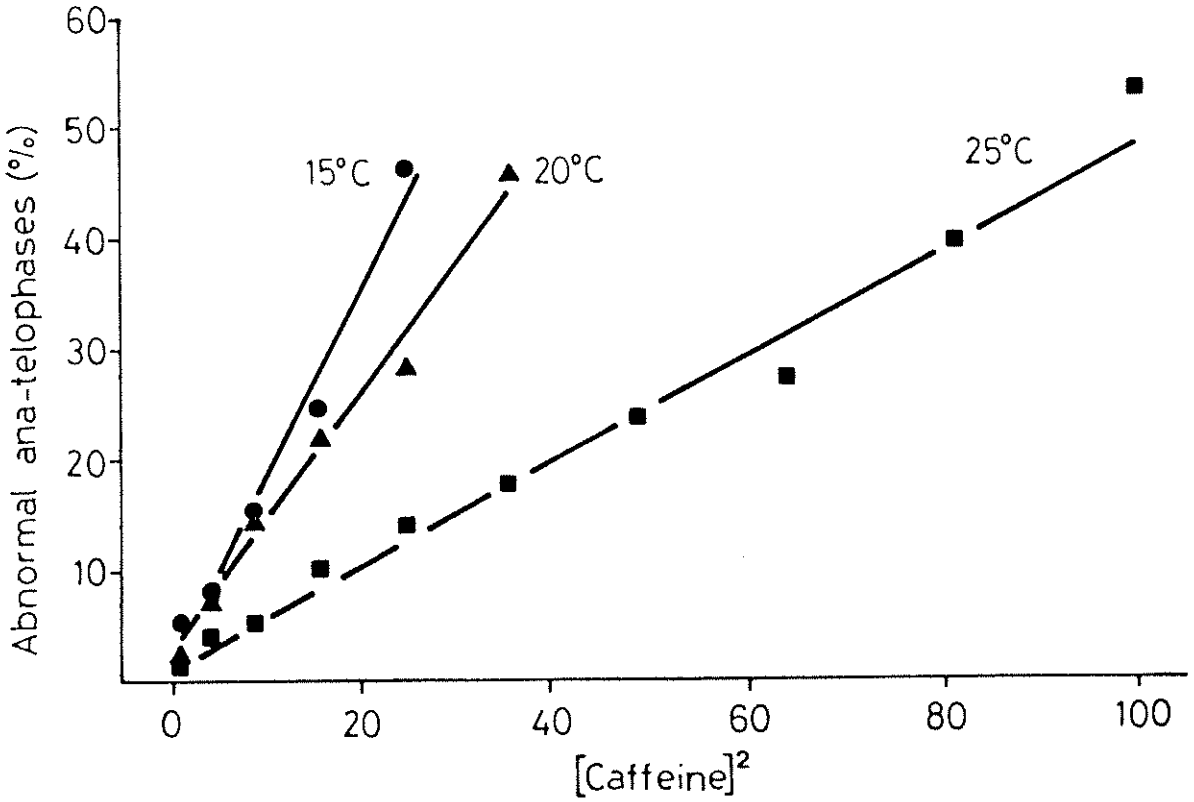

Fig. 9.- Percentage of abnormal ana-telophases plotted against the square of caffeine concentration under three different growth temperatures. Abnormal ana-telophases were measured at the end of caffeine treatment. Correlation coefficients were 0.996, 0.986 and 0.995 for 15, 20 and 25 °C, respectively.

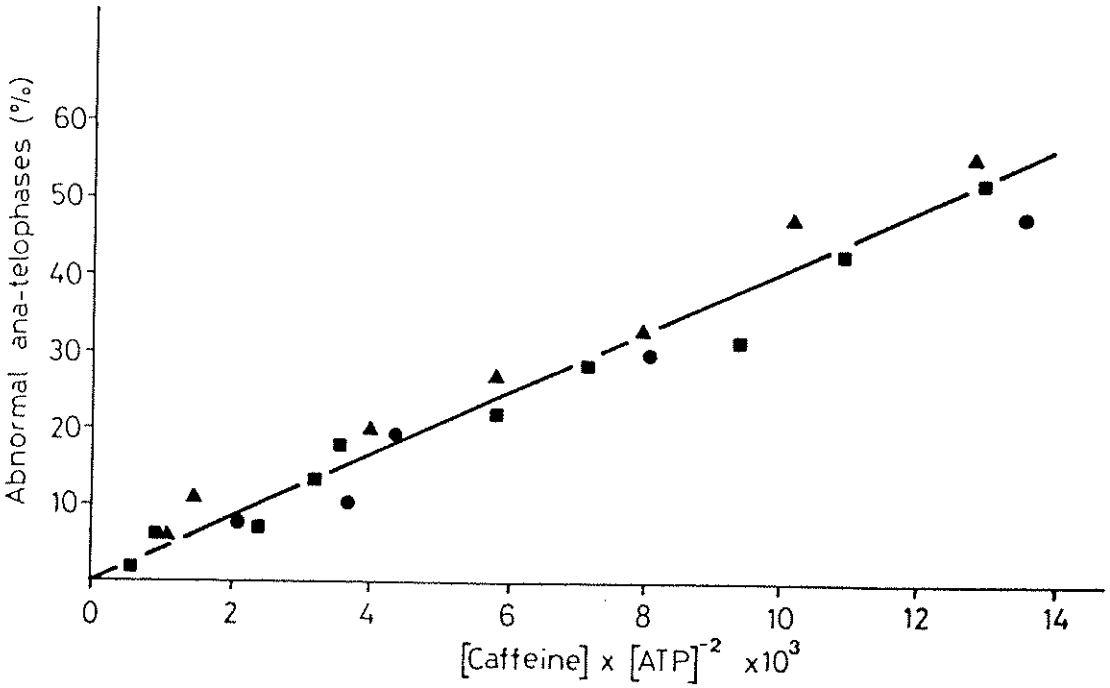

Fig. 10.- Correlation between caffeine-induced abnormal ana-telophases and [caffeine] x $[ATP]^{-2}$ under different growth temperatures, 15, 20, 25 °C.

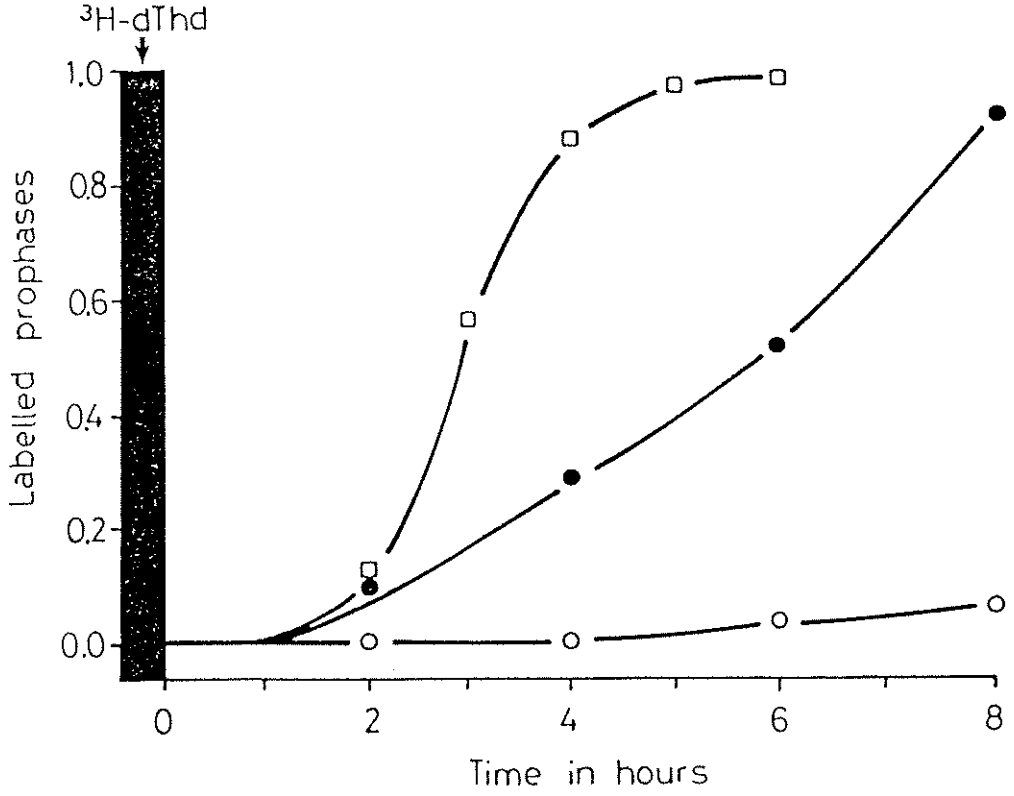

Fig. 11.- Frequency of labelled prophases after a 20-min pulse with [^{3}H] thymidine under control conditions (□); when the ^{3}H-thymidine pulse was followed by a 1-h treatment with 2 mM methyl-methane sulfonate (MMS) (○) and when, after the [^{3}H] thymidine pulse, the roots were incubated for 1-h in 2 mM MMS and post-treated with 2 mM caffeine until the end of the experiment (●).

Table 5.- ATP levels (pmoles/μg protein) observed at different growth temperatures after caffeine treatment for similar cycle time periods.

Caffeine concentration (mM)	Growth temperature (°C)		
	15	20	25
0	20.5	29.6	38.9
1	23.0	31.4	46.6
2	26.0	43.8	68.0
3	32.0	25.0	31.6
4	24.2	22.9	32.0
5	18.0	20.6	35.8
6	NT	19.1	25.0
7	NT	NT	24.0
8	NT	NT	19.5
9	NT	NT	19.0
10	NT	NT	16.7

NT, not tested

Table 6.- Estimate of chromosome damage and G_2 duration after a tritiated thymidine pulse followed by different treatments

Treatment (3 h)	Postreatment (2 h)	Abnormal[a] anatolephase (%)	Duration of G_2 (h)	Mitotic delay (h)
–	–	0.5	2.8	–
0.5 mM AU	–	16.4	6.4	3.6
–	1μg/ml Anisomycin	0.3	3.2	0.4
0.5 mM AU	3 mM Caffeine	46.8	4.6	1.8
0.5 mM AU	3 mM Caf. + 1 g/ml Anison.	7.3	5.3	2.5
0.5 mM AU	3 mM Caf. + 0.5 g/ml Cyclohex.	26.7	NT	NT
0.5 mM AU	0.5μ/ml Cycloheximide	12	NT	NT

a) The abnormal ana-telophases were analysed 5 h after addition of ^{3}H-dThd.
NT, not tested

with AU-treated cells. Thus, 3 mM caffeine doubles the chromosome damage in MMS-treated cells and cancels a great part of the important delay induced by the alkylating agent.

Effect of cycloheximide on G2 repair

In mammalian cells, Das et. al, (1982) and Lau and Pardue, (1982) demonstrated the abolition by cycloheximide of caffeine enhanced lethality of alkylating agents and proposed that caffeine does not act directly, but rather by means of some newly-synthesized protein(s). Thus. HN2-treated cells were protected from the lethal and chromosome-damaging effects of caffeine by low doses of cycloheximide. It has been presumed by some authors that proteins might be required for normal mitosis and that these could be induced by caffeine (Roberts, 1984a and 1984b).

At low concentrations, which inhibit about a fifty per cent of the rate of protein synthesis in our experimental system, cycloheximide and anisomycin have been able to ameliorate the G2 repair after AU or MMS incubations. When some of them was added during caffeine post-treatment, AU-treated and MMS-treated cells were protected substantially, as shown in the Table 6 and 8. Even more, cycloheximide can strikingly reduce the chromosome damage induced by 9 mM caffeine on control cells (Table 7) and can also improve the G2 repair of AU-treated cells in the absence of caffeine (Table 6).

When the estimation of G2 duration was carried out, in anisomycin experiments (Table 6), this protein synthesis inhibitor has, in every case, lengthened the G2 duration in relation with its control condition. For instance, G2 of control cells lasted 2.8 h and anysomicin incubation of 2 h induced a mitotic delay of 0.4 h AU-treated cells presented 6.4 h, caffeine reduced this duration to 4.6 h and the combined treatment with caffeine and anysomicin showed 5.3 h of G2 period (Similar experiments with cycloheximide are going now on in our lab).

As a consequence, it seems logical to assume that cycloheximide is able to ameliorate DNA repair mechanisms, which operate at different moments of the cell cycle, even in the presence of caffeine. Likely, inhibition of protein synthesis during the post-treatment with damaging agents allows damaged cells to have enough time for DNA repair before to go on in their cell cycle.

G2-Prophase mechanism for chromosome repair: a postulate

Most of the DNA damaging agents affect cellular proliferation in a similar way: the block damaged cells in the premitotic G2 period of the cell cycle. Depending of the dose, this G2 arrest is reversible and damaged cells undergo mitotic delay. Interestingly, in some cases, even if cells located in previous cell cycle phases (G1 or S) are exposed to these agents, they suffer a transient stop when they reach G2 phase (Tomasovic and Dewey, 1978; Kimler et al, 1982). Some of these damaging agents are ionizing radiation, alkylating agents and DNA synthesis inhibitors. Therefore, *G2 arrest can be considered as a general response of proliferating cells to DNA damage.*

Tobey (1975) has postulated the existence of a surveillance mechanism in G2 that prevents highly damaged cells from entering mitosis and proliferating further. Consistently with these ideas, Hittelman & Rao (1974) have shown, using premature chromosome condensation, that cells accumulated in G2 because of exposure to alkylating agents in fact possess a large number of chromosome breaks.

In front of a broad range of G2 arrest-inducing treatments (heat shocks, starvations, inhibitors and mutagens) a single pleiotropic mechanism appears to mediate the transient physiological response of the cells (Herrlich et all., 1984).

In fact, many authors have obtained evidence suggesting that a DNA-repair mechanism exists which operates during G2 and prophase, repairing DNA lesions. When this mechanism

Table 7.- Percentage of abnormal ana-telophases and mitotic index induced by caffeine alone and caffeine plus cycloheximide[a]

Treatment (2 h)	Mitotic index (%)	Abnormal ana-telophases (%)
Control	12.8	0.0
9 mM Caffeine	10.6	56.9
0,5μg/ml cycloheximide	7.7	2.2
9 mM Caffeine + 0.5μg/ml Cycloheximide	5.0	25.7

a) Both parameters were scored at the end of the treatment.

Table 8.- Percentage of abnormal anatelophases and mitotic index induced by methylmethanesulfonate (MMS) alone or followed by different treatments[a]

Treatment (1 h)	Post-treatment (3 h)	Mitotic index (%)	Abnormal anatelophases (%)
2 mM MMS	–	6.3	14.4
2 mM MMS	3 mM Caffeine	8.4	33.6
2 mM MMS	3 mM Caf. + 0.5μg/ml Cyclohex.	5.3	4.3

a) Both parameters were scored 3 h after the MMS incubation.

fails or makes mistakes chromosomal aberrations may be produced (Taylor et al., 1962; Kihlman and Hartley, 1968; Yamamoto and Yamaguchi, 1969; Natarajan et al., 1980; Hartley-Asp et al., 1980; Preston, 1980; Palitti et al., 1981; Kihlman et al., 1982b).

Recently, De Marco and Polani (1981), González-Fernández and López-Sáez (1982), Kihlman et al. (1982a) and González-Fernández et al. (1985) have postulated that, during late G2 and prophase, chromatin condensation could lead to the induction of chromosome damage from pre-existing lesions which would be readily repaired by a G2-prophase repairing mechanism. When the frequency of lesions that reach G2 has been increased by any clastogenic agent and/or this chromosome repairing is inhibited chromosomal aberrations are strongly increased in next mitosis.

We would like to propose that the G2-prophase repair mechanism can be composed of two cooperative pathways:

1) *Mitotic delay* (G2 arrest) associated with chromosome damage and
2) *DNA repair pathways* for removing lesions and restoring original structure of DNA.

Mitotic delay is generally associated with a certain damage induced in DNA by clastogenic agent. As a rule, the greater the potential of any agent to induce damage on DNA, the stronger the depression in cell cycle transit and, when this damage is subsequently expressed as chromosomal aberrations, the larger the G2 delay (Rao, 1980).

Apparently, G2 period not only serves to prepare cells for mitosis, but also appears as a cycle phase for cell recovery (Yamada and Puck, 1961; Tobey, 1975; and Lücke-Hüle, 1982). Protein synthesis, but not RNA synthesis, is required for recovery from G2 arrest.

Sunkara et al. (1979) have demonstrated that mitotic factors, able to regulate chromosome condensation and nuclear membrane breakdown during mitosis, accumulate slowly in the beginnig of G2 but a progressively more rapid during late G2 and reach a threshold at the G2-mitosis transition when chromatin condenses into chromosomes. During metaphase, when the chromosomes are most condensed, the levels of mitotic factors are the highest. The mitotic factors, which are non-histone proteins, have a great affinity for chromatin and preferentially bind to it as soon as they are synthesized in G2 period (Adlakha et al., 1982).

We propose that division delay produced after induction of DNA damage results from unavailability or inactivation of proteins essential for chromosome condensation and mitosis development. The results recently obtained by Adlakha et al., (1983) support this suggestion. They demonstrated the presence of *Inhibitors of Mitotic Factors* in mammalian cells during G1, but not in S or G2 cells. Moreover, extracts of quiescent(G0) human fibroblasts exhibited very little inhibitory activity, but u.v. irradiation of Go cells significantly enhaced the inhibitory activity of these extracts. These factors seem to be activated, rather than newly synthesized, either when cells enter telophase or when they are irradiated (Addlakha et al., 1984).

Therefore, we propose that DNA lesions, probably single-stranded areas, are recognised by an *inhibiting factor* (IF). This process activates IF producing an IF + which then inactivates *mitotic factors* bringing about the G2 arrest.

This mechanism must involve a magnifying action, for a low number of lesions is able to induce the response. In our model, the G2 arrest would be characterized by a continuous synthesis of mitotic factor and a concurrent inactivation of it while the inhibiting factor remains activated, as IF+. When DNA repair has removed the lesions, restoring the structure of the native conformation with low or negligible activity against mitotic factor and the continuous synthesis of mitotic factor allows the cell to go into mitosis.

DNA-repair pathaways, during the G2 period, require DNA precursors and DNA synthesis and ligation, making the whole process sensitive to inhibitors such as 5-fluorodeoxyuridine, cytosine arabinoside, hydroxyurea, 3-aminobenzamide, etc. (Taylor et al. 1962; Kihlman and Hartley, 1968; Cohen, 1977; Preston, 1980; Palitti et al. 1981; Das et al. 1984; Schubert et al., 1986).

Thus, in HBK cells arrested in G2 after a mild treatment with HN2 unscheduled DNA synthesis could be detected (Lau and Pardee, 1982). Sometimes, the failure of DNA inhibitors to prevent DNA rejoining is probably due to the exiguous amount of DNA synthesis that accom-

panies this process. However, 5 mM hydroxyurea is able to potentiate X-ray-induced chromosomal aberrations in human lymphocytes during G2, likely by reducing the supply of material required for repair (Hansson et al., 1982).

Poly (ADP-ribose) polymerase is a nuclear enzyme which catalyses the synthesis of poly (ADP-ribose) from the ADP-ribose moiety of NAD + (Hilz and Stone, 1976) and inhibitors of this enzyme, e.g. 3-aminobenzamide, have been found to prevent the rejoining of strands breaks in DNA (Durkacz et al., 1980). Recently, it has been shown that ADP-ribosylation is probably involved in the regulation of the activity of DNA ligase II (Creissen and Shall, 1982).

At the chromosomal level, post-treatments with 3-aminobenzamide in G2 enhanced the frequency of X-ray-induced chromatid aberrations in Chinese hamster cells (Natarajan et al. 1982), while in BHK and human lymphocytes they have no potentiating effect on the yield of chromatid aberrations induced by alkylating agents (Das et al., 1984; Hansson et al., 1984).

Besides, in yeast cells and in human leukocytes treated with several cytotoxic agents, it has been demonstrated that DNA repair correlates with the rate ot ATP-production in a sigmoid manner (Jain et al., 1982; Verma et al., 1982). These observations are in agreement with the demonstration that the rate of DNA, RNA and protein synthesis are ATP level dependent (Sims et al., 1983).

Lastly, we can point out that a number of adenine nucleotide derivatives have been associated to DNA repair such as ATP and NAD+, above mentionated, and 2-5-oligoadenilates, diadenosine 5-5 p p tetraphosphate (Ap 4A) and cAMP (see Herrlich et al., 1984, for references).

Molecular action of caffeine on the G2 repair mechanism

This caffeine effect has features characteristic of methyl xanthines at the cellular and molecular level: a rapid entrance into the cell, an easy removal by dilution, and partial reversion by adenosine derivatives (for references see González-Fernández and López-Sáez, 1980). These facts suggest that the affinity for its target must be very weak.

Cancellation of G2 arrest. It is a well known fact that caffeine post-treatments cancel division delay induced by physical or chemical agents. Generally, the division delay is dose dependent, e.g. the greater the DNA damage the longer the delay.

In an important work, Kimler et al. (1982) have demonstrated that the *relative cancellation* of radiation-induced division delay by caffeine, theophylline and theobromine is dependent on the methyloxypurine concentration and apparently independent on the radiation dose, between 100-300 rad in Chinese hamster ovary cells.

Tomasovic and Dewey (1978) in animal cells and our group in plant cells (González-Fernández et al., 1985) have demonstrated that adenosine does not modify, or only marginally reduces, the caffeine effect on mitotic delay. Thus, the G2 arrest or its cancellation appear to be non-dependent on cellular ATP level.

To explain in a simple way the molecular action of methyl xantines on division delay we would like to propose that these drugs are able to inhibit in a dose dependent manner the delay by affecting the activation of the *inhibiting factor*. Unfortunately, we cannot yet make any discussion on the binding site. It may be the DNA lesions, the inhibiting factor, the complex DNA-IF or its activity, but somehow the presence of these drugs would stabilize the *mitotic factors*, committing cells with damaged DNA to go into mitosis.

Inhibition of DNA repair pathways. While caffeine cancellation of division delay can be considered a "hard" fact, any effect of this drug on the rate of DNA repair during G2 and prophase remains to be proven. However, a number of experimental results suggests caffeine can inhibit some step(s) of DNA repair pathways, slowing down the rate of repair and/or inducing misrepair in a dose dependent manner.

Besides, the potentiating action of caffeine treatments on damaged cells can be partially abolished by adenosine, without affecting the caffeine action on division delay (González-Fernández et al., 1985).

Therefore, we postulate that the caffeine action reversed by adenosine can be a certain slowing down in the rate of DNA repair. As many other caffeine actions, this effect can involve competition with some adenine nucleotide derivative (s).

In Chinese hamster cells, Cremer et al. (1980) have demonstrated that the strong potentiating effect of UV-light plus caffeine was significantly reduced, if the post-treatment was performed with caffeine plus the four deoxyribosides. Probably, these precursors for DNA synthesis can improve the rate of repair.

Recently, it has been proposed that ataxia telangiectasia (AT) cells, more sensitive than normal cells to ionizing radiation, are not reversibly blocked in G2 but die there. (Ford et al., 1984). Perhaps, the high level of DNA damage that reaches G2 when increased by ionizing radiation can induce a suicide mechanism, as it has been proposed by Sims et al, (1983). In this particular case, the G2 period would be no more a period for cell recovery but a period for cell to die.

Acknowledgements

This work was partially supported by the Comisión Asesora de Investigación Científica y Técnica, and by a research agreement between CSIC (Spain) and the University of Chile. The authors thank M.L. Martínez, M.C. Partearroyo and M. Carrascosa for technical assistance.

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