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The Effects of Treatments with 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide in Darkness and in Light on Survival and Progression in Chinese Hamster Ovary Cells *in Vitro*[1]

A. H. Gerulath,[2] S. C. Barranco and R. M. Humphrey

Department of Obstetrics and Gynaecology, University of Toronto, St. Michael's Hospital, 30 Bond Street, Toronto M5B 1W8, Ontario Canada [A. H. G.]; Department of Human Biological Chemistry and Genetics, Division of Cell Biology, University of Texas Medical Branch, Galveston, Texas, 77550 [S. C. B.]; and Department of Physics, Section of Cellular Studies, University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas, 77025[R. M. H.]

SUMMARY

The cytotoxic action of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide in Chinese hamster ovary cells is enhanced considerably in the presence of light, when 5-diazoimidazole-4-carboxamide is being continuously generated. A broad shoulder threshold-type survival curve is seen when the cells are exposed to 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (both in light and dark).

The drug had only slight effects on the survival of cells treated in the dark for short times throughout the cell cycle. However, cells in the G_1 and early S phases were slightly more sensitive when treated with the drug in the light. Both light and dark treatments with the drug caused progression delay in S phase. Treatment in the light delayed cells at the G_2-M boundary, whereas dark treatments delayed cells at the S-G_2 boundary. Both light and dark treatments with the drug caused some G_1 cells to progress into S phase 1 hr early, while another fraction of the same population was delayed in G_1 phase.

INTRODUCTION

DIC,[3] a new antineoplastic agent used primarily in the treatment of malignant melanoma, was developed in the search for new inhibitors of *de novo* purine synthesis (8). The exposure of DIC to light results initially in the formation of DZC which is cytotoxic to bacteria and mammalian cells (6, 8). This conversion of DIC by light *in vitro* may in some way mimic the activation of the drug *in vivo*. In this paper, we describe the effects of DIC treatments in darkness and in light on cell survival, cell cycle stage sensitivity, and progression kinetics in CHO cells *in vitro*.

MATERIALS AND METHODS

Cells and Culture Technique. A CHO cell line was grown in monolayer cultures as described by Humphrey *et al.* (5). The average cell cycle time under these conditions was 13 hr with a 2.5-hr G_1 phase, an 8-hr S phase, and a 1.5-hr G_2 period. All experiments were performed on cells initially in the logarithmic phase of growth. Plating efficiency ranged from 80 to 90%. In testing the cytotoxic action of the drug, survival was determined by the ability of the treated cells to form colonies. Known numbers of single cells were plated into plastic Petri dishes and incubated for 6 to 8 days at 37°. The colonies that formed were fixed, stained with crystal violet, and counted. A cell was considered to have obtained reproductive capacity (to be viable) if it gave rise to a colony of 50 or more cells. All experiments were performed 3 times. The graphs represent the average of 9 plates/point for survival determinations and 3 plates/point for cell progression studies.

Synchronization of Cells. To obtain large numbers of cells synchronized at the beginning of the S period, a double treatment with excess TdR was used, as described by Humphrey *et al.* (4).

The degree of synchrony was monitored by pulse-labeling cells at various times with TdR-^{3}H (1 μCi/ml, 1.9 Ci/mmole) to determine (by autoradiography) the percentage of cells in S phase, and by scoring the MI. At the end of the synchrony procedure, approximately 95% of the population was in early S phase and progressed into G_2 and mitosis in a synchronous fashion.

In experiments requiring the treatment of cells in G_1 phase, synchronized cells were obtained by using the excess TdR block followed by selection of mitotic cells (12). The resulting MI ranged from 92 to 97%. The mitotic cells were plated into dishes containing fresh, warm medium and allowed to progress into G_1 phase. The degree of synchrony was monitored in each experiment by scoring the MI that

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[3] The abbreviations used are: DIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DZC, 5-diazoimidazole-4-carboxamide; CHO, Chinese hamster ovary; TdR, thymidine; LI, labeling index; MI, mitotic index.

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fell to 0% within 1 hr after plating. Cells were pulse-labeled at various times thereafter with TdR-^{3}H and the number of labeled cells was scored. One thousand cells were scored for each sample.

Autoradiography Techniques. In all experiments involving the assay of populations of labeled cells, Ilford K5 liquid emulsion autoradiograph techniques were used. A cell was considered labeled if it contained at least 10 grains over the nucleus. Less than 3 grains were observed in background areas of similar size.

Progression of Asynchronous Cells. In order to distinguish between the mitotic figures of cells that had been treated in G_2 and in S phases, asynchronous CHO cell populations were pulse labeled with TdR-^{3}H (1 μCi/ml; 1.9 Ci/mmole) for 10 min, washed twice, and then treated with DIC dissolved in media. The treatment medium was removed and the cells were washed twice with fresh medium containing nonradioactive TdR (10 μg/ml). Colcemid (0.06 μg/ml) was added to the Petri dishes, and the dishes were returned to the incubator. At 20-min intervals thereafter for 4 hr, samples were taken. Cells were removed from the dishes with 0.025% trypsin, centrifuged, fixed in 50% glacial acetic acid, and stained with acetoorcein. Slides for autoradiography were prepared and exposed for 2 weeks, and the percentage of labeled and unlabeled cells in metaphase accumulated in Colcemid was determined by scoring 1000 cells.

Preparation of DIC. DIC was supplied through the courtesy of Dr. Ti Li Loo by the Drug Development Branch, Drug Research and Development, National Cancer Institute. DIC solutions were made up in the desired concentrations immediately prior to use. A stock solution of 20,000 μg/ml was made in 0.1 N HCl and kept at 4°. This was diluted into medium to the proper treatment concentrations. The pH of the treatment medium was adjusted to pH 7.2 to 7.4 The decomposition of DIC in medium follows 1st-order kinetics with a half-life of 34 hr in the dark and 3.3 hr in the light (2). In all light experiments, a (Model P-2324 fluorescent lamp equipped with two 15-watt Ken-Rad F15T8/CW bulbs, Dazor Manufacturing Corporation, St. Louis, Mo.) was placed in the incubator 38 cm from the cells. In the dark experiments, the cells were shielded from light by wrapping the culture flasks in aluminum foil prior to placement in the incubator. The drug was added in a dark room.

RESULTS

Survival of CHO Cells (Asynchronous). The survival fraction of cells following continuous exposure for different time intervals to various concentrations of DIC are shown in Chart 1 (treatment in darkness) and Chart 2 (treatment in light). Little effect was observed following short treatment times of 6 hr in the dark (Chart 1) or 4.5 hr in the light (Chart 2). Cell lethality increased as the drug concentration and duration of treatment increased and, in all cases, it was shown that exposure of cells to the drug in the presence of light caused greater cell lethality than did the dark treatments. Exposure of cells to DIC in the dark (Chart 1) resulted in broad-shoulder or threshold-type survival curves, exhibiting exponential decreases in survival with longer treatment times. The shoulder becomes smaller with increasing treatment time, but is still substantial in the populations treated for 48 hr. A 24-hr exposure to DIC (1000 μg/ml) (in the dark) reduced the survival fraction to 12% (Chart 1). A 48-hr exposure to the same drug concentration resulted in a survival fraction of 1%.

The cytotoxic effect of DIC treatment in light is more pronounced (Chart 2). Little difference between survival data was observed after exposure times of 12 and 24 hr. Following a 24-hr exposure to 500 μg DIC/ml (light), the survival fraction was reduced to 3.5%. Treatment with 1000 μg/ml for 24 hr (light) decreased the survival fraction to 0.0015% (Chart 2).

Survival of CHO Cells Treated in M and G_1 Phases. In all experiments involving survival data from synchronized cells, the position of the cells in the cell cycle at the time of treatment was determined from TdR-^{3}H LI's or by determining the percentage of cells in mitosis. The survival

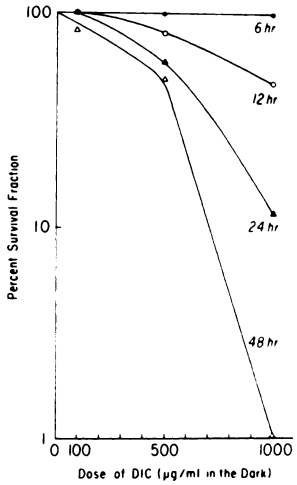

Chart 1. The survival fractions of CHO cells following treatment with various concentrations of DIC (in the dark) for different lengths of time (6, 12, 24, or 48 hr).

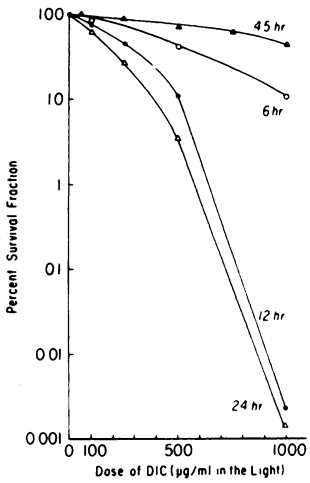

Chart 2. The survival fractions of CHO cells following treatment with various concentrations of DIC (in the light) for different lengths of time (4.5, 6, 12, and 24 hr).

fractions of synchronized cells treated in mitosis and in the 1st and 2nd hr of G_1 phase are shown in Chart 3.

Because of the possibility of cells progressing out of M or G_1 phase during treatment, a high drug concentration (1000 μg/ml for 1 hr) was used in the following experiments. Mitotic and G_1 cells treated with DIC in the dark were only slightly sensitive to the drug (90% survival fractions). However, increased cell lethality was observed in cell populations treated in the light. Survival of cells treated initially in mitosis or in the 1st hr of G_1 phase was 62 and 72%, respectively. Cells treated during the 2nd hr of G_1 phase were more sensitive, with survival being reduced to 23% following the 1-hr treatment.

Survival of Cells Treated in S and G_2. Cells in S phase (synchronized by the excess TdR method) were exposed to a 2-hr treatment with DIC (1000 μg/ml). The effect of DIC on cells treated in the dark is very minimal, resulting in a survival fraction of 73% in early S phase (Chart 4).

Cell survival increases through mid-S, with the least effect seen in late S and G_2, where 100% of cells survive the DIC treatment in the dark. S and G_2 cells exposed to DIC in the light (Chart 4) had sensitivities quite similar to those of late-G_1 cells, but only if the DIC treatment was for 2 hr instead of the 1 hr of exposure used on the G_1 cells. Sensitivity in early S phase reached a minimal survival fraction of 20%, whereas survival in mid-, and late S phase and G_2 phase was higher.

Cell Progression after DIC Exposure in Mitosis. Populations of cells synchronized in mitosis were treated with 1000 μg DIC/ml (continuous treatment). At the end of 1 hr, the MI was 1% in the control and treated plates both in darkness and light. Therefore, treated cells were not delayed

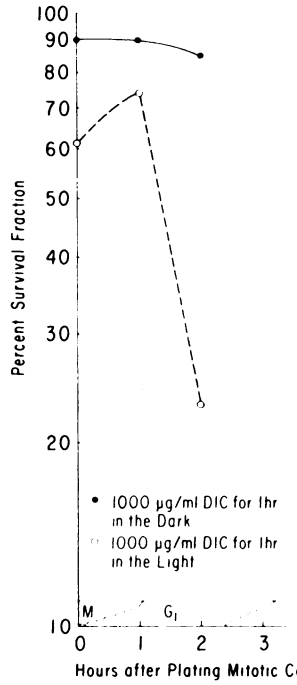

Chart 3. The effect of DIC on survival of synchronized CHO cells treated in mitosis and G_1 phases of the cell cycle.

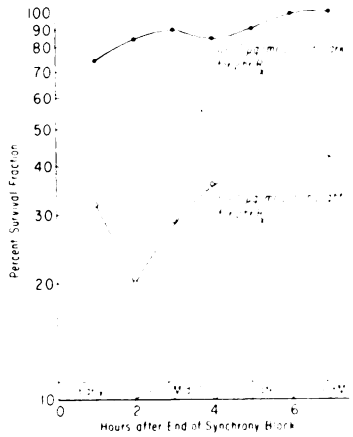

Chart 4. The effect of DIC on survival of synchronized CHO cells treated in S and G_2 phases of the cell cycle. R_x, treatment.

in mitosis and progressed from M into G_1 at the same rate as the control population (data not shown).

Progression after DIC Exposure during the G_1 Phase. Mitotic cells (95% MI) were plated into warm medium and allowed to progress into G_1. One hr after plating, the MI had fallen from 95 to 0% and the cells were a well-synchronized G_1 population. The labeled fraction in the control cell population began increasing linearly 2 hr after cells were plated, thus indicating cell progression from G_1 into S (Chart 5). The labeled control cells peaked at 6 hr and then decreased as the cells passed through S into G_2 and mitosis.

Replicate plates of cells were treated at a point 1 hr into G_1 phase, in either the dark or the light with DIC (1000 μg/ml) (continuous treatment). Approximately 40% of cells treated with DIC in darkness and 50% of cells treated in the light entered the S phase 1 hr early (Chart 5). Since the percentage of labeled cells in both the dark and light populations did not increase to the level of the control cells, this would suggest that although some cells progressed into S phase early, another fraction of cells were delayed in G_1 phase by the DIC treatment.

Progression after DIC Exposure during the S Phase. Populations of cells synchronized in early S phase by an excess TdR block were treated with DIC (1000 μg/ml) (continuous treatment). It can be seen (Chart 6) that the percentage of cells labeled with TdR-^{3}H was 97% in the control population 1 hr after release from the synchrony treatment. The LI fell to a minimum of 30% between 6 and 7 hr as the cells moved out of S and into G_2 and mitosis. As the percentage of labeled control reaches a minimum, the MI reaches a peak value of 13% at 7 hr. This is a characteristic, reproducible phenomenon of the TdR synchrony technique and allows the determination of progression changes in treated populations throughout the S phase.

Most of the cells treated with DIC both in darkness and in light were delayed in progressing through and out of the S phase. Initially, most treated cells were delayed 1 hr in S; at 4 hr, the LI in the treated cells was unchanged at 90% compared with 60% in the control. By 5 hr, the LI in the treated cells had decreased to 80% as a small percentage of cells overcame the block; the LI remained at this level until 8 hr when a 2nd decrease occurred, falling to 50% in the case

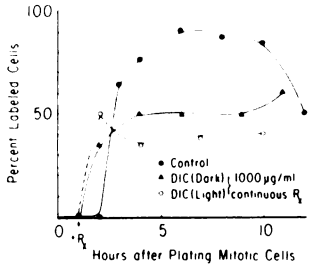

Chart 5. DIC effect on cell progression from G_1 phase into S phase.

of cells treated in darkness and to 63% in cells treated in light.

Only a small percentage of treated cells were able to progress normally out of S and into G_2 and M, reaching a mitotic peak of 1% (light) and 2% (dark) at the same time that the control population had a MI of 13%.

Progression after DIC Exposure in the G_2 Phase. By pulse labeling asynchronous populations of cells with TdR-^{3}H immediately before drug treatment and scoring the percentage of labeled and unlabeled cells progressing into the metaphase, it is possible to determine whether a progression delay has occurred in cells in S or G_2 phase at the start of treatment. Chart 7 shows that the percentage of unlabeled control cells in the metaphase (accumulated in Colcemid) increased linearly with time for 3.5 hr. Unlabeled metaphases are still seen in control populations at 3.5 hr instead of plateauing in number at 2.5 hr (length of G_2 phase) because all cells do not progress through the cell cycle at the same rate. The number of labeled metaphase cells (cells in S phase at the time of the TdR-^{3}H pulse label) does begin to increase at 2.5 hr, however (data not shown). Cells exposed continuously to DIC (1000 μg/ml) in the presence of light are delayed in progressing into mitosis. There is no return to a normal progression rate during the sampling period. Cells treated with DIC (1000 μg/ml) in darkness progress normally for 2.3 hr when a delay occurs. In the latter case it is evident that cells in mid- and late G_2 are not affected by the drug treatment, whereas cells in early G_2 (probably at the S-G_2 boundary) are prevented from progressing into M.

DISCUSSION

The cytotoxic action of DIC is enhanced considerably in the presence of light, confirming previous observations in CHO and melanoma cells (2). The survival fraction observed in cells following 24 hr of exposure to DIC (1000 μg/ml) in the light was 0.0015% as compared to 12% survival following the treatment in the dark. The photodecomposition of DIC results in the formation of DZC and dimethylamine (7). DZC then undergoes irreversible cyclization to form the biologically inert 2-azahypoxanthine. The light experiments, therefore, primarily reflect the action of DZC, which is being continually generated; however, the possibility of combination effects caused by DIC, DZC, and dimethylamine cannot be ruled out. Experiments carried out in the dark represent the activity of DIC.

Asynchronous cells exposed to DIC (in the dark and light) exhibit broad-shoulder threshold-type survival curves, with smaller shoulders seen in cells treated in the light. This indicates either that sublethal damage may have occurred, and that there is a threshold dose below which no cell killing is observed, or that a proportion of cells has recovered from sublethal cell damage (possibly through a repair process). Of course other pharmacological mechanisms, such as drug activation, membrane exclusion, and drug breakdown, may be the cause of the shoulder. Such studies are continuing in our laboratories.

The treatments of synchronized mitotic and G_1 cells were for only 1 hr because the durations of these phases are short and because of the possibility that the cells would progress through the cell cycle during the treatment. However, S phase is 8 hr long in the CHO cells, and treatments of 2 hr duration were used. Although direct absolute comparisons of drug sensitivity in these phases cannot be made because of the differences in treatment duration, relative differences in survival after treatment are obvious. DIC is only moderately effectual in killing cells in early S, as 73% of cells survive treatment in the dark; a lesser effect is seen in mid-S, and marked cell resistance to the drug is observed in the other phases of the cell cycle. In contrast, DIC treatment in the light kills approximately 3 times the number of cells in G_1 (1-hr treatment) and early S (2-hr treatment).

Both dark and light treatments with DIC prevent the progression of cells from S phase to G_2 and mitosis. This suggests interference with DNA synthesis. Whether this interference occurs through inhibition of *de novo* purine synthesis, as was originally postulated (8), or another

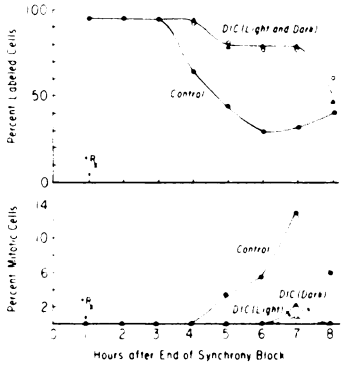

Chart 6. Effect on cell progression from S phase to G_2 and mitosis during continuous treatments with DIC (1000 μg/ml).

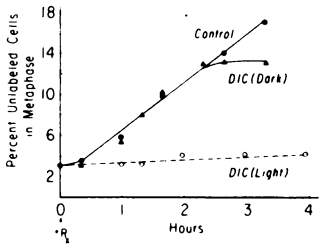

Chart 7. Effect on cell progression from G_2 phase into mitosis during continuous treatments with DIC (1000 μg/ml).

mechanism, is a matter of conjecture. *In vivo* studies in the rat have suggested that DIC selectively inhibits DNA but not RNA and protein synthesis (11). Similarly, DZC primarily inhibits DNA synthesis in *Escherichia coli* (14) and *Bacillus subtilis* (6), but not RNA and protein synthesis. An interaction of DZC with DNA of CHO cells has recently been demonstrated (2).

It must be emphasized that the photodecomposition reaction of DIC with liberation of the highly active DZC may have no counterpart *in vivo*. In fact, DIC in darkness is thought to undergo oxidative demethylation to 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MIC) with the ultimate formation of 5-aminoimidazole-4-carboxamide and a methyl carbonium ion (2). The latter, which attacks DNA, is presumably responsible for the antitumor actions of DIC *in vivo* (3, 10).

The results described in this paper suggest the possibility that DZC (DIC in the light) interferes with RNA and/or protein synthesis throughout the cycle, resulting in a cell progression delay. Its interaction with RNA in CHO cells has been documented (2), in contrast to findings in bacteria (6, 14).

The progression delay of S phase cells into G_2 and mitosis could be caused by drug interference at the translational level. In spite of continuous treatment, this block was overcome after several hr; the latter effect may be partly a reflection of drug decay.

Cells exposed continuously to DIC (light) in G_2 were unable to enter mitosis. This drug thus appears to interfere with the synthesis or function of a "division-specific protein," necessary for the initiation of mitosis (1, 13). In contrast, DIC (dark) caused a progression delay only in those cells in early G_2 (probably at the S-G_2 boundary), thus possibly indicating a different locus or mechanism of action. A DIC-induced G_2 progression delay has also been demonstrated in leukemia L1210 cells (9). In this cell system, however, RNA and protein synthesis were affected more than DNA synthesis.

Under the influence of DIC (in darkness and in light) some cells progressed from G_1 into S 1 hr early. The explanation for this is unclear; however, stimulation of macromolecular synthesis in *B. subtilis* has been reported with low concentrations of DIC and DZC (6). Most of the cells in our experiments were delayed in G_1 and were prevented from entering S. It thus appears that higher drug concentrations inhibit RNA and/or protein synthesis. These findings agree with *in vitro* results utilizing leukemia L1210 cells (9) but are at variance with bacterial studies (6, 14).

For therapeutic considerations, it is evident that DIC kills cells most effectively in G_1 and early S phase. It is only moderately lethal to cells in other phases of the cell cycle. The drug causes progression delay in G_1, S, and early G_2 cells.

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REFERENCES

1. Barranco, S. C., and Humphrey, R. M. A Time Specific Effect of UV-Irradiation on Cells Progressing from G_2 into Metaphase. Mutation Res., *9:* 524-526, 1970.
2. Gerulath, A. H., and Loo, T. L. Mechanism of Action of 5-(3,3-Dimethyl-1-Triazeno)Imidazole-4-Carboxamide in Mammalian Cells in Culture. Biochem. Pharmacol., *21:* 2335-2343, 1972.
3. Housholder, G. E., and Loo, T. L. Disposition of 5-(3,3-Dimethyl-1-Triazeno)Imidazole-4-Carboxamide, a New Antitumor Agent. J. Pharmacol. Exptl. Therap., *179:* 386-395, 1971.
4. Humphrey, R. M., Sedita, B. A., and Meyn, R. E. Recovery of Chinese Hamster Cells from Ultraviolet Irradiation Damage. Intern. J. Radiation Biol., *18:* 61-69, 1970.
5. Humphrey, R. M., Steward, D. L., and Sedita, B. A. DNA Strand Scission and Rejoining in Mammalian Cells. *In:* Genetic Concepts and Neoplasia. M. D. Anderson Hospital and Tumor Institute at Houston, pp. 570-592. Baltimore: The Williams & Wilkins Co., 1970.
6. Saunders, P. P., and Schultz, G. A. Studies of the Mechanism of Action of the Antitumor Agent 5(4)-(3,3-Dimethyl-1-Triazeno)Imidazole-4(5)-Carboxamide in *Bacillus subtilis*. Biochem. Pharmacol., *19:* 911-920, 1970.
7. Shealy, Y. F., Krauth, C. A., and Montgomery, J. A. Imidazoles. I. Coupling Reactions of 5-Diazoimidazole-4-Carboxamide. J. Org. Chem., *27:* 2150-2154, 1962.
8. Shealy, Y. F., Montgomery, J. A., and Laster, W. R. Antitumor Activity of Triazenoimidazoles. Biochem. Pharmacol., *11:* 674-676, 1962.
9. Shirakawa, S. and Frei, E., III. Comparative Effects of the Antitumor Agents 5-(Dimethyltriazeno)-imidazole-4-carboxamide and 1,3-Bis (2-chloroethyl)-1-nitrosourea on Cell Cycle of L1210 Leukemia Cells *in Vivo*. Cancer Res., *30:* 2173-2198, 1970.
10. Skibba, J. L., Beal, D. D., Ramirez, G., and Bryan, G. T. *N*-Demethylation of the Antineoplastic Agent 4(5)-(3,3-Dimethyl-1-triazeno)-imidazole-5(4)-carboxamide by Rats and Man. Cancer Res., *30:* 147-150, 1970.
11. Skibba, J. L., Ertürk, E., and Bryan, G. T. Effects of 4(5)-(3,3-Dimethyl-1-Triazeno)Imidazole-5(4)-Carboxamide (NSC 45388) in Proliferating Rat Tissues. Biochem. Pharmacol., *21:* 2817-2824, 1972.
12. Terasima, T., and Tolmach, L. J. Changes in X-Ray Sensitivity of HeLa Cells during the Division Cycle. Nature, *190:* 1210-1211, 1961.
13. Tobey, R. A., Petersen, D. F., and Anderson, E. C. Energy Requirements for Mitosis in Chinese Hamster Cells. *In:* R. Baserga (ed.), Biochemistry of Cell Division, pp. 39-56. Springfield, Ill.: Charles C Thomas, Publisher, 1969.
14. Yamamoto, I. 4(or 5)-Diazimidazole-5(or 4)-Carboxamide and Related Triazenoimidazoles as Antibacterial Agents: Their Effects on Nucleic Acid Metabolism of *Escherichia coli B*. Biochem. Pharmacol., *18:* 1463-1472, 1969.