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STUDIES OF THE MECHANISM OF ACTION OF THE ANTITUMOR AGENT 5(4)-(3,3-DIMETHYL-1-TRIAZENO) IMIDAZOLE-4(5)-CARBOXAMIDE IN *BACILLUS SUBTILIS**

PRISCILLA P. SAUNDERS and GLORIA A. SCHULTZ

Departments of Pathology and Developmental Therapeutics, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025, U.S.A.

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Abstract—The effect of 5(4)-(3,3-dimethyl-1-triazeno) imidazole-4(5)-carboxamide (NSC 45388; DIC) on *Bacillus subtilis* has been investigated under various conditions. The exposure of DIC to light prior to its addition to bacterial cultures markedly decreased its lethality, presumably as a result of its conversion of 2-azahypoxanthine. However, exposure to light of cultures growing in the presence of DIC dramatically enhanced the inhibitory activity of the agent, compared to identical cultures growing in the dark. 4-Diazoimidazole-5-carboxamide, the immediate photodecomposition product of DIC, was subsequently tested and found to be highly inhibitory for *B. subtilis*. Amino acids were found to enhance the inhibitory activity of both DIC and the diazo compound. DIC inhibition of growth could be reversed by the addition of reduced glutathione. A DIC-resistant strain of *B. subtilis* was isolated and found to be resistant also to the diazo compound. Studies on the effect of DIC on macromolecular syntheses indicated that the drug slightly stimulated DNA, RNA and protein synthesis at low concentrations and inhibited DNA synthesis at higher levels.

THE ACTIVITY of several triazenoimidazoles against neoplastic and other cells has been demonstrated in a number of systems, both *in vivo* and *in vitro*.[1–3] More recently, it has been shown by Luce and Thurman[4] that this type of compound is significantly active in human melanoma. The specific mechanism of action of these compounds has, however, remained obscure. It is the purpose of this communication to clarify the mechanism of one of these drugs, 5(4)-(3,3-dimethyl-1-triazeno) imidazole-4(5)-carboxamide (NSC 45388; DIC), by presenting evidence relevant to its active form in bacterial systems.

Shealy *et al.*[5] have demonstrated that the exposure to light of certain disubstituted triazenes, such as DIC, results in the formation of 2-azahypoxanthine (II). This transformation (Fig. 1) presumably results from a light-catalyzed dissociation of the triazene to 5-diazoimidazole-4-carboxamide (I) and dimethylamine (Fig. 1). The diazo compound (I) can then undergo irreversible cyclization to form 2-azahypoxanthine (II). The light-catalyzed decomposition of DIC has been employed by Loo and Stasswender[6] in a specific colorimetric assay for this class of compounds. The relevance of these reactions in a biological system will be considered in this report.

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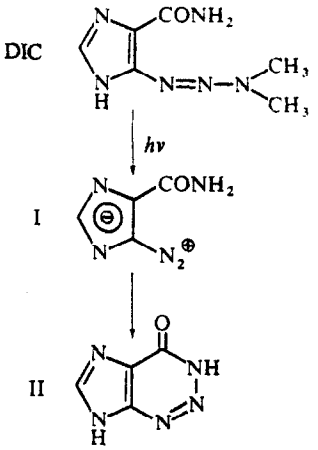

FIG. 1. Light-catalyzed decomposition of DIC.

MATERIALS AND METHODS

Bacillus subtilis 168 was employed as the experimental organism. A minimal medium previously described[7] was used in all experiments. The isolation of a DIC-resistant strain of 168, 168-DIC^{R+}, was accomplished by increasing stepwise the concentration of the drug in minimal medium. An aliquot of the culture was plated onto minimal agar and a single colony was picked and checked for DIC resistance.

Growth experiments were carried out in a New Brunswick water bath shaker with a stainless steel cover (when desired) at 37°. Nephelometer flasks, 250 or 500 ml, were usually employed. Absorbancy measurements were made on a Bausch & Lomb Spectronic 20 colorimeter at 420 mμ.

For routine growth experiments, cells were grown to an absorbancy of approximately 0·35 and divided into portions of 10–15 ml. The desired additions (drugs, etc.) were made and incubation was continued for 2–3 hr. Absorbancy readings were made as desired. Viable cell counts were determined as previously described.[7] The methods used in the colorimetric determinations of the synthesis of DNA, RNA and protein have been previously described.[7]

DIC (NSC 45388) was obtained from the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute. 4-Diazoimidazole-5-carboxamide (I) was provided by Dr. Ti Li Loo. "Vitamin-free" casein hydrolysate (enzymatic) was purchased from Nutritional Biochemicals Company.

RESULTS AND DISCUSSION

Effect of DIC on growth of B. subtilis. Figure 2 shows a family of short-term growth curves resulting from exposure of logarithmically growing cultures to varying levels of DIC. This technique was found to produce the most reproducible effects of DIC and was, therefore, used as an assay of inhibitory activity in all experiments described. It is interesting to note that in nearly every growth experiment 60 min was required before the effects of the drug became pronounced.

Effect of DIC previously exposed to light. In view of the photodecomposition of DIC,[5] it seemed pertinent to determine whether the inhibitory activity of the drug could be attributed to either of the products of this reaction, 4-diazoimidazole-5-

carboxamide (I) or 2-azahypoxanthine (II). A simple means of determining the relative effectiveness of the latter was carried out by exposing a sample of DIC to light (KEN-RAD F 15T8/CW fluorescent desk lamp) overnight. The DIC was approximately 10 cm from the light source. Colorimetric analysis[6] of DIC solutions treated in this way indicated that the conversion of DIC to 2-azahypoxanthine was complete. This assay detects both DIC and the diazo compound. The product of this treatment was then tested for inhibitory activity in comparison to DIC that had not been exposed to light. Figure 3 shows that light treatment markedly reduced the activity of the drug, thus indicating that 2-azahypoxanthine is not the active form of the drug in this system.

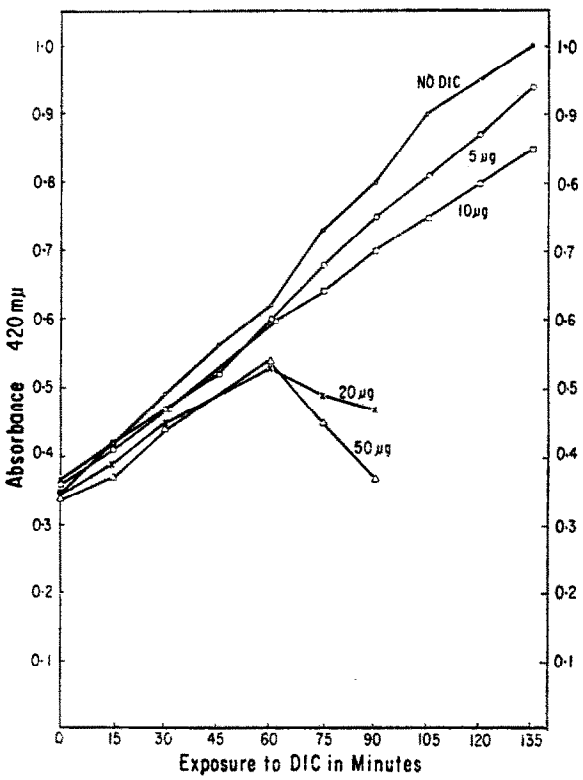

FIG. 2. Effect of varying levels of DIC on growth of *B. subtilis* 168. Numbers refer to μg/ml of DIC Incubation was carried out in the dark.

Effect of DIC in the presence and absence of light. To test the activity of the diazo compound, an experiment was carried out in which one set of cultures was incubated in direct light (40 cm from a fluorescent desk lamp), while another was incubated in a covered water bath shaker. Thus, in those cultures exposed to light, the diazo compound was continually being generated. As shown in Fig. 4A, DIC inhibition was dramatically enhanced in the presence of light. These observations can be interpreted to indicate that in bacteria the diazo compound is either the primary active form of DIC or a direct precursor of the active form.

In order to determine the effect of the drug on cell viability in the light, viable

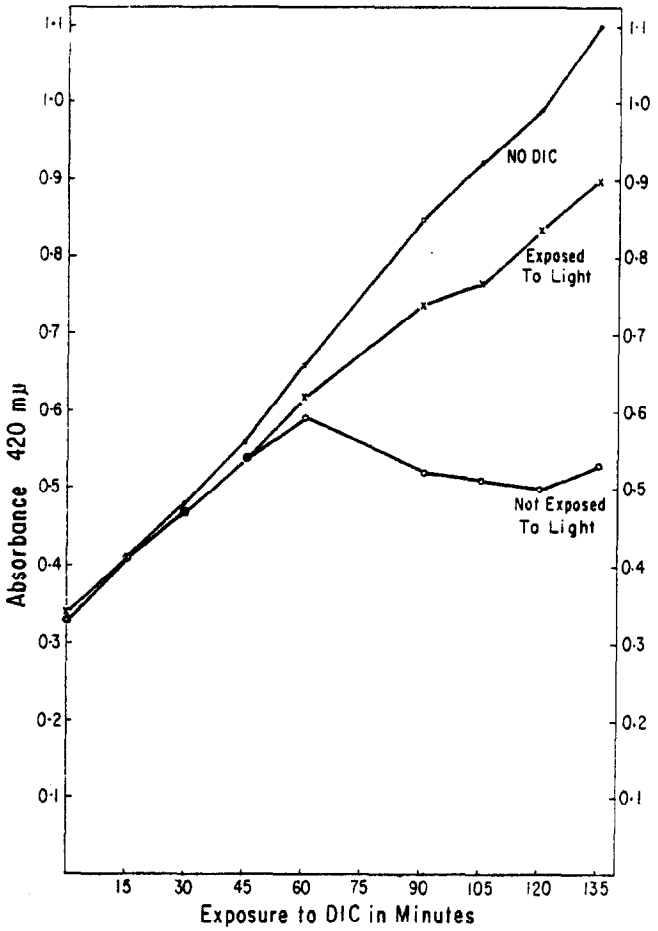

FIG. 3. Effectiveness of DIC (20 μg/ml) previously exposed to light. Strain 168 was employed and incubation was carried out in the dark.

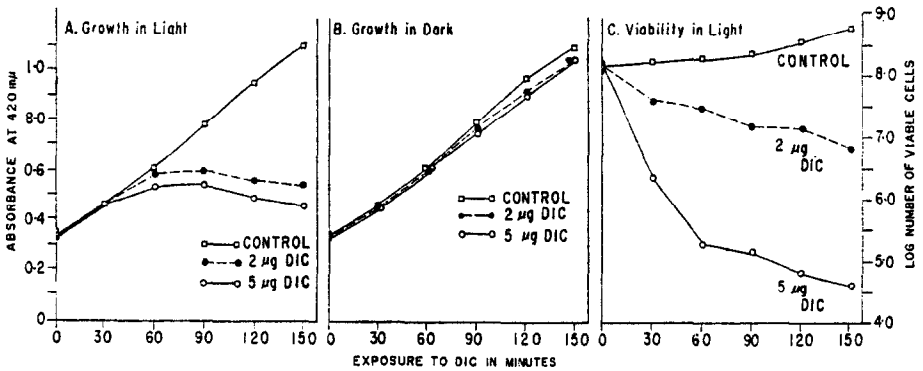

FIG. 4. Effect of DIC on viability (C) and growth of *B. subtilis* 168 in the light (A) and in the dark (B). Growth was measured by absorbance. Numbers refer to μg/ml of DIC.

counts were made by plating samples, throughout the growth curve, onto a complete medium (Fig. 4C). DIC at the higher concentration appeared to be immediately lethal to the cells, even though the absorbancy increased somewhat before declining.

Effect of DIC on a resistant strain (168-*DIC*$^{R+}$). The effect of DIC on *B. subtilis* 168-DIC^{R+} (right panel) compared to the wild type (left panel) is shown in Fig. 5. The resistant strain showed normal growth at levels of DIC (5–10 μg/ml) which were highly inhibitory to strain 168.

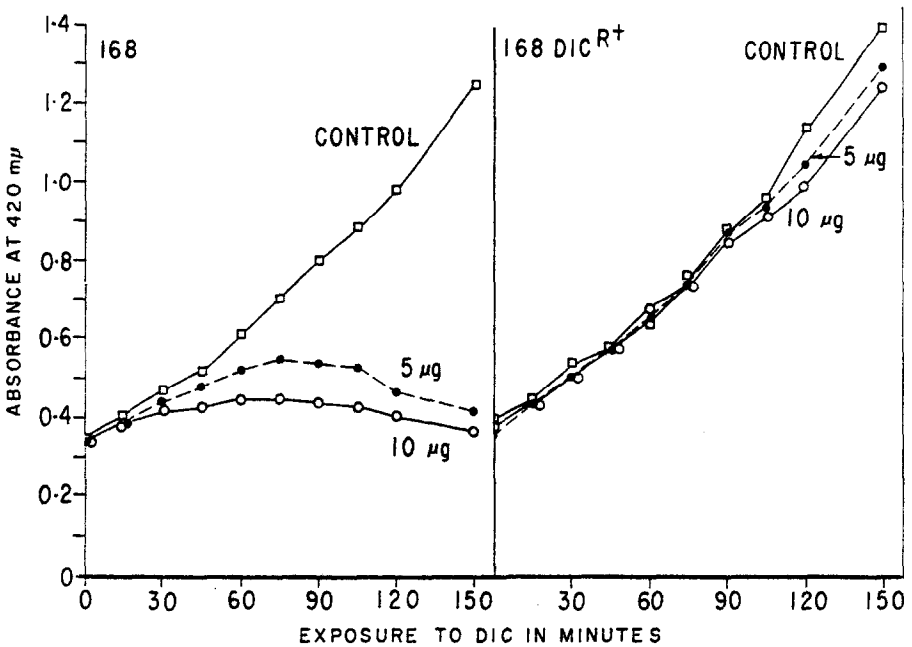

FIG. 5. Effect of DIC on growth of strains 168 and 168-DIC^{R+}. Numbers refer to μg/ml of DIC. Incubation was carried out in the light.

Effect of 4-diazoimidazole-5-carboxamide. The possibility that the diazo compound is the active form of DIC was further tested by incubating *B. subtilis* directly with the compound. Although it has been reported to cyclize rapidly to 2-azahypoxanthine, 4-diazoimidazole-5-carboxamide does effectively inhibit *B. subtilis*, as shown in Fig. 6. Light, which is necessary only for the formation of the diazo compound from DIC,[5] had little or no influence. The DIC-resistant strain was also tested for sensitivity to the diazo compound. Here again, although both organisms were inhibited at the higher levels of the compound, the DIC-resistant strain showed a conspicuous tolerance in each case. These observations provide further evidence for attributing the inhibitory activity of DIC to the formation of 5-diazoimidazole-4-carboxamide.

Effect of amino acids on DIC inhibition. An interesting effect was observed by the addition of amino acids to the cultures at the same time that the drug was introduced. Instead of reversing the effect of the drug, as might have been expected for histidine (structural similarity), the addition of the amino acid enhanced the inhibitory activity

of DIC (Fig. 7). Additional experiments showed that amino acids in general had this effect. Similar experiments with the diazo compound showed the same phenomenon (Fig. 8).

Effect of glutathione on DIC inhibition. In studying the biological activity of diazonium compounds, Iwata *et al.*[8] observed interactions between 4-diazoimidazole-5-carboxamide and certain sulfhydryl groups in tissues. Since the diazo compound appears to be the active form of DIC in bacterial systems, one might expect nontoxic sulfhydryl compounds to reverse DIC inhibition. Figure 9 shows that this is indeed the

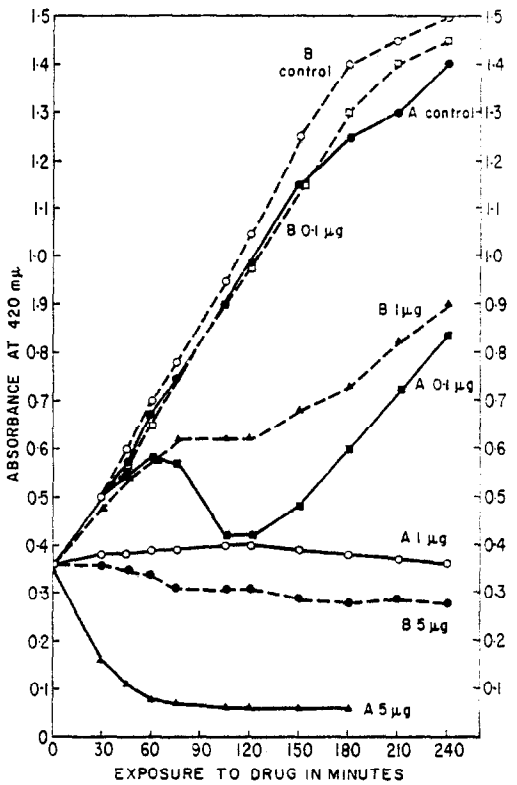

FIG. 6. Effect of 5-diazoimidazole-4-carboxamide on growth of strains 168 (A) and 168-DIC^{R+} (B). Numbers refer to μg/ml of the diazo compound.

case, although relatively high levels of reduced glutathione were required. Although it will be necessary to examine the chemistry involved to conclude that this is the result of direct interaction between the diazo compound and the sulfhydryl group of glutathione, it is probable that this is the case.

Effect of DIC on synthesis of macromolecules. In an attempt to assign a primary site of action to DIC, experiments were conducted to determine the effect of the drug on the synthesis of cellular DNA, RNA and protein. An experiment was carried out in which the synthesis of the various macromolecules was measured colorimetrically. To

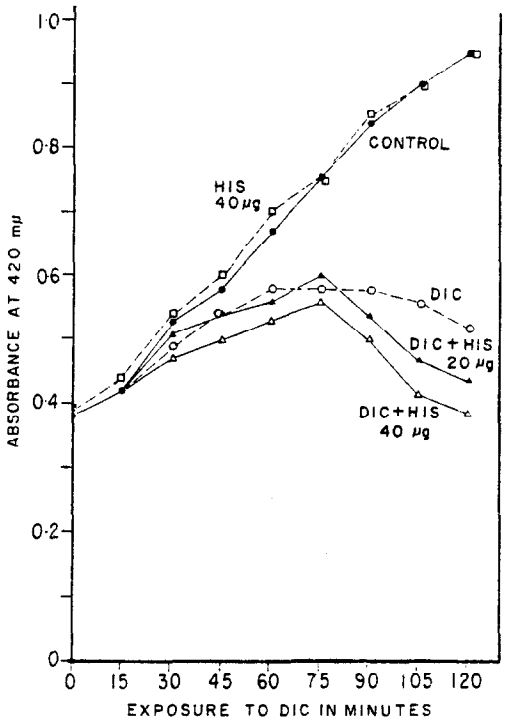

FIG. 7. Effectiveness of DIC (25 μg/ml) in combination with histidine. Incubation of strain 168 was carried out in the dark.

TABLE 1. EFFECT OF DIC ON PROTEIN, RNA AND DNA SYNTHESIS

Exposure to DIC (min)	No DIC				DIC (3 μg/ml)				DIC (10 μg/ml)			
		(μg/ml/0·1 O.D.)				(μg/ml/0·1 O.D.)				(μg/ml/0·1 O.D.)		
	A_{420}*	Protein	RNA	DNA	A_{420}	Protein	RNA	DNA	A_{420}	Protein	RNA	DNA
0	0·37	13·6	2·9	0·40	0·37	13·6	2·9	0·40	0·37	13·6	2·9	0·40
60	0·65	14·7	3·1	0·42	0·58	16·0	3·2	0·45	0·54	13·1	3·1	0·24
90	0·8	18·1	3·7	0·47	0·62	19·9	3·7	0·50	0·56	15·4	3·0	0·24
120	0·95	18·1	3·4	0·48	0·56	22·3	4·4	0·63	0·52	16·2	3·2	0·19
150	1·10	22·2	3·9	0·52	0·62	26·3	5·2	0·69	0·42	18·1	3·7	0·23

* Absorbancy at 420 mμ of a 5-ml sample was read on a Bausch & Lomb Spectronic 20.

enhance the inhibition, incubation was carried out in the light (40 cm from a fluorescent desk lamp).

The data of Table 1 are expressed as micromoles of each macromolecule per amount of cell mass (as measured by absorbancy) at various time intervals. Thus, the numbers should be relatively constant throughout the logarithmic phase of growth. The increases (particularly in RNA and protein) in the control probably reflect the onset of the stationary phase of growth. From Table 1 it is clear that, at the lower level of DIC, protein, RNA and DNA synthesis proceeded at normal or slightly

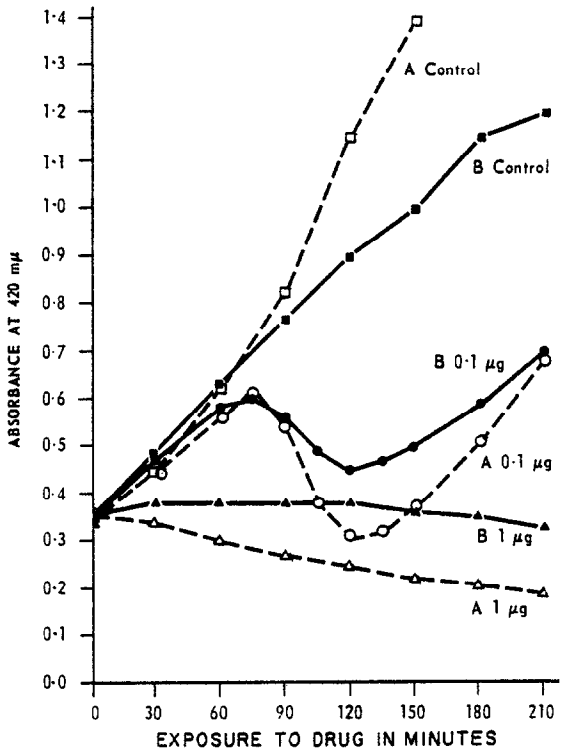

FIG. 8. Effectiveness of 5-diazoimidazole-4-carboxamide with (A) and without (B) casein hydrolysate (2·5 mg/ml). Incubation of strain 168 was carried out in the light. Numbers refer to μg/ml of the diazo compound.

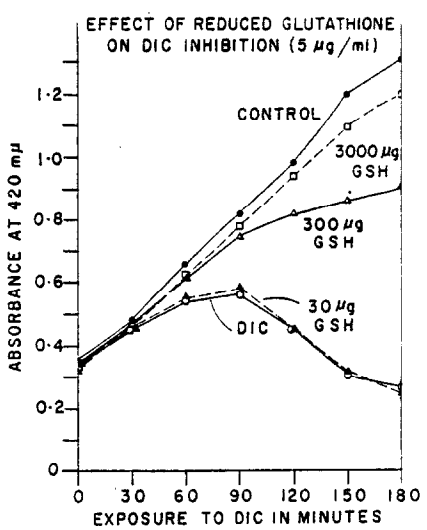

FIG. 9. Effect of glutathione (GSH) on DIC (5 μg/ml) inhibition of growth. Numbers refer to μg/ml of reduced glutathione. Incubation of strain 168 was carried out in the light.

stimulated rates even though growth was somewhat inhibited. This is consistent with isotope incorporation experiments which were carried out on another strain of *B. subtilis*. At the higher level of DIC, DNA synthesis was, however, markedly inhibited, while RNA and protein synthesis continued. There was also an indication of some DNA degradative activity as the cells began to lyse. Thus it appears that, although DNA synthesis cannot be considered the primary site of action, it is affected at high enough levels of the drug. Further experimentation on a biochemical level will be necessary to explain this observation.

All of the data presented here serve to emphasize the importance of 5-diazoimidazole-4-carboxamide in considerations of the mechanism of action of DIC and related compounds. Since initial growth inhibition cannot be attributed to interference with macromolecular synthetic processes, it seems quite possible that DIC may be acting as an alkylating agent, thus producing a general cytotoxic effect. The ease of coupling of the diazo compound with sulfhydryl groups (and perhaps other reactive groups as well) could also be of primary significance in the inhibitory mechanism of the drug. While it is conceivable that more than one mode of action is involved in DIC inhibition, it is probable that the diazo compound is involved in at least one of them. Work is in progress to determine: (1) if there are other inhibitory mechanisms not involving the diazo compound; and (2) if so, which is the most significant in a biological system.

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