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

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Abstract—The inhibitory activity of 5(4)-(3,3-dimethyl-1-triazeno)imidazole-4(5)-carboxamide (DIC) in *Bacillus subtilis* has been shown to be dependent on the presence of light during incubation. 4-Diazoimidazole-5-carboxamide, the immediate photodecomposition product of DIC, was subsequently tested and found to be highly inhibitory. Uptake experiments with DIC-2-^{14}C indicate that the diazo compound rather than DIC itself enters the cells. The presence of reduced glutathione in the growth medium retards the uptake of isotope. DIC is lethal to both proliferating and non-proliferating cells. Experiments *in vitro* have been carried out indicating a light-catalyzed association of DIC-2-^{14}C with certain purified enzymes.

TRIAZENOIMIDAZOLES have been shown to be active against neoplastic and other cells both *in vivo* and *in vitro*.[1–3] Hano *et al.*,[2] working with a series of 4(5)-dialkyltriazenoimidazole-5(4)-carboxamides, have shown a correlation between the antitumor activity (Ehrlich carcinoma) of the drug and the length of the alkyl chains. An increase in the dialkyl chain length caused a decrease in the tumor-inhibitory effect. Luce and Thurman[4] have shown that 4(5)-dimethyltriazenoimidazole-5(4)-carboxamide (DIC) is significantly effective against human melanoma. Evidence has also been presented indicating that 5(4)-aminoimidazole-4(5)-carboxamide (AIC) is excreted in abnormally large amounts in the urine of patients receiving DIC therapy.[5–7]

A previous communication from this laboratory[8] has presented evidence which strongly suggests that the active form (or precursor of the active form) of DIC in bacteria is 4-diazoimidazole-5-carboxamide (Compound I in Fig. 1), the immediate photodecomposition product of DIC. The observations supporting this conclusion were: (A) the presence of light during incubation was necessary for inhibition of growth, (B) the diazo compound was tested and found to be highly inhibitory with no requirement for light, and (C) 2-azahypoxanthine (Compound II in Fig. 1), the product of spontaneous cyclization of the diazo compound, is not significantly inhibitory. Growth inhibition by DIC could be reversed by the addition of reduced glutathione or enhanced by the addition of amino acids. Yamamoto[9] has presented similar evidence regarding the activity of 4-diazoimidazole-5-carboxamide in *Escherichia coli.*

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Iwata *et al.*[10] have shown that the diazo compound is an effective inhibitor of milk xanthine oxidase. Suggestive evidence has also been presented indicating that the diazo compound interacts with sulfhydryl groups in tissues.[11]

This communication will describe experiments with isotopically labeled DIC relating to the fate of the agent in *Bacillus subtilis* cells.

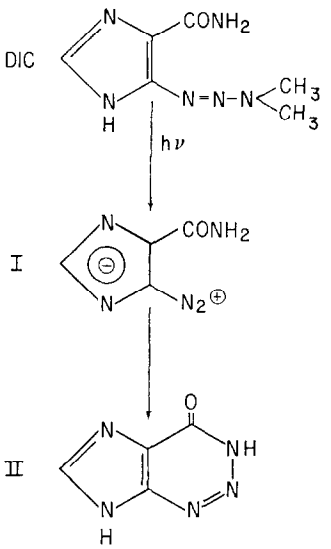

FIG. 1. Light-catalyzed decomposition of DIC to 4-diazoimidazole-5-carboxamide (I) and 2-aza-hypoxanthine (II). Another product, dimethylamine, is not shown.

MATERIALS AND METHODS

B. subtilis 168 was employed as the experimental organism. A minimal medium previously described[12] was used in all experiments. Absorbancy measurements were made on either a Bausch and Lomb spectronic 20 colorimeter or a Beckman model DU-2 spectrophotometer. For routine growth and isotope uptake experiments, cells were grown in a water bath shaker at 37° to an absorbancy at 420 nm 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. To measure isotope uptake 1·0-ml samples were removed at appropriate time intervals, the cells collected directly onto 25 mm type B6 (Schleicher & Schuell Co., Keene, N.H.) membrane filters, and washed several times with 5-ml portions of cold minimal medium. The filters were then dried and counted for 5 min in a Packard Tri-carb liquid scintillation spectrometer. The counting solution was toluene containing 0·01% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene and 0·4% 2,5-diphenyl-oxazole. After 30 min the samples were filtered, washed several times with cold medium, and counted as previously described.[13]

Viable cell counts were made by appropriately diluting samples of the cultures and plating onto a complete medium as previously described.[12] Colonies were counted after 18 hr incubation at 37°.

In experiments requiring non-proliferating cells, a culture of strain 168 was grown to early log phase as usual, harvested on a millipore filter, washed and resuspended in the same medium lacking tryptophan which is required for growth by strain 168. After 30 or 60 min incubation to allow the cells to utilize any endogenous tryptophan, the desired additions were made and incubation continued. Samples were withdrawn and treated as above to obtain the viable counts.

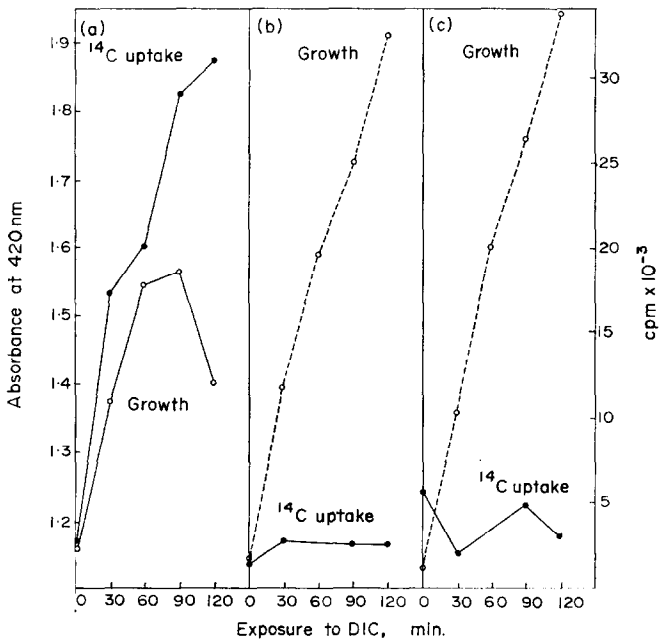

FIG. 2. Uptake of isotope from DIC-2-^{14}C by *B. subtilis*. Cultures were grown to an absorbance at 420 nm of 0·35 as described in Methods, harvested by centrifugation and concentrated by resuspending in one-third the original volume of medium. Added was 0·18 μc/ml DIC-2-^{14}C(3·3 μg/ml) and the cultures were incubated and treated as described in Methods. (a) and (c) were incubated in the light. (b) was incubated in the dark. (c) contained 1·5 mg/ml GSH.

When incubation was carried out in the light, a KEN-RAD F 15T8/cw fluorescent desk lamp was used as the light source approximately 40 cm above the shaking culture flasks. Experiments in the dark were executed in a water bath shaker with a stainless steel cover.

2-Azahypoxanthine-^{14}C was prepared by exposing a sample of DIC-2-^{14}C (1 mg/ml H_2O, 54·5 μc/mg) to light (KEN-RNA F 15T8/cw fluorescent desk lamp) overnight. The DIC solution was approximately 10 cm from the light source. Colorimetric analysis[14] of the solution indicated that the conversion of DIC to 2-azahypoxanthine was complete. This assay detects both DIC and the diazo compound.

DIC (NSC-45388) and DIC-2-^{14}C were obtained from the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute. 5-Fluorouracil (FU) was purchased from CalBiochem. Crystalline lysozyme and horse liver alcohol dehydrogenase were obtained from Worthington Biochemical Corp. 4-Diazoimidazole-5-carboxamide was furnished by Dr. T. L. Loo.

RESULTS AND DISCUSSION

Uptake of isotope from DIC-2-^{14}C by growing B. subtilis

In view of the aforementioned evidence that 4-diazoimidazole-5-carboxamide is the active form of DIC, it was of interest to determine whether or not DIC itself enters the cell. Cells were incubated with DIC-2-^{14}C and samples were removed; the cells were washed and the isotope was counted. Figure 2 (A, B) shows that growing *B. subtilis* cells appeared to take up isotope from DIC-2-^{14}C provided incubation was carried out in the light (A). The limited uptake in the dark (B) is suggestive that it is

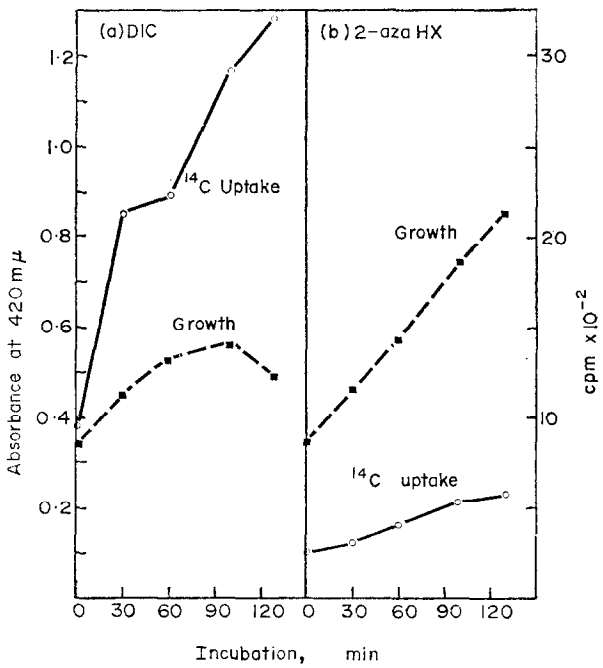

FIG. 3. Uptake of 2-azahypoxanthine-^{14}C by *B. subtilis*. Cultures contained 1·1 μc DIC-2-^{14}C (a) or 2-azahypoxanthine-^{14}C (light-treated DIC-2-^{14}C) (b). Incubation and treatment of the cells were as described in Methods.

not DIC itself that is taken up but rather one of the photodecomposition products of DIC (Fig. 1), 4-diazoimidazole-5-carboxamide (I) or 2-azahypoxanthine (II). Since the cultures which were incubated in the dark received brief exposures to light when absorbancy readings were made, it is quite possible that the small amount of uptake that did occur in (B) reflects that fraction of DIC which decomposed as a result.

If the isotope that was taken up by the cells was in the form of 2-azahypoxanthine, the cells should accordingly take up DIC-2-^{14}C which had previously been exposed to light such that neither DIC nor the diazo compound remained in the solution. This was accomplished as described in Methods, and the experiment shown in Fig. 3 was carried out. Very little uptake of the isotope in this form was observed. This allows the conclusion that the isotope that became associated with the cells in Fig. 2

cannot be attributed to the formation of 2-azahypoxanthine. Therefore, it appears that the isotope that became associated with the cells under these conditions was in the form of 4-diazoimidazole-5-carboxamide. It is possible that 2-azahypoxanthine was also taken up and was subsequently washed out of the cells under the conditions employed. 2-Azahypoxanthine will not, however, be considered further in this report since it is not inhibitory to *B. subtilis* at the concentrations used here.[8]

Since 4-diazoimidazole-5-carboxamide is a highly reactive compound, the possibility must be considered that the isotope from ^{14}C-DIC that appeared to be taken up by the cells could merely reflect an interaction between this compound and the cell wall. An experiment was carried out (Table 1) to determine the relative amounts

TABLE 1. CELLULAR LOCALIZATION OF ISOTOPE FROM ^{14}C-DIC*

Compound used to label cells	Counts per min in original extract	Counts per min in isolated cell wall fragments	Counts per min in cell wall / total counts per min (%)
^{3}H-DAP	23,580	2690	11·3
^{3}H-thymidine	402,380	8810	2·1
^{14}C-DIC	112,770	530	0·4

* Twenty-ml cultures of strain 168 were grown to an absorbance at 420 nm of 0·5 followed by the addition of 2 μc/ml ^{3}H-DAP (200 mc/m-mole), 2 μc/ml ^{3}H-thymidine (10 c/m-mole) or 0·28 μc/ml ^{14}C-DIC (15·5 mc/m-mole). Incubation was continued for 2 hr in the light as previously described after which the cells were collected by centrifugation and washed twice with cold minimal medium. To each batch of labeled cells was added 500 mg wet weight of unlabeled cells as carrier followed by suspension in 10 ml of cold distilled water. The cells were disrupted by four 10-sec bursts with a Branson Sonifier model 185-C. Intact cells were removed by centrifugation at 500 *g* for 5 min and again for 10 min. Samples of the extract were removed for counting. Cell wall fragments were then obtained by centrifugation of this extract at 10,000 *g* for 10 min and washed 3 times with 10 ml of cold distilled water. The cell wall fragments were then resuspended in 1 ml of the same, and samples were counted, after drying onto glass fiber filters, in the solvent previously described.

of isotope associated with isolated cell walls and in the cell cytoplasm. ^{3}H-diaminopimelic acid (^{3}H-DAP), a cell wall precursor,[15] and ^{3}H-thymidine were included as controls. Growing cultures were incubated with each of the isotopes for 2 hr after which the cells were harvested, washed and disrupted. Those cell wall fragments that were large enough to be removed from the solution by centrifugation at 10,000 *g* for 10 min were harvested and washed as described. Of the isotope from ^{3}H-DAP that was taken up by the cells, 11 per cent was found in the isolated cell wall fragments while only 0·4 per cent of the isotope from ^{14}C-DIC was in the cell wall fragments. While it appeared that 2 per cent of the thymidine was in the cell walls, this probably represents residual DNA that was associated with the cell walls. It is possible that some of the cell wall fragments were small enough to have remained in solution. However, the comparison of the percentages of ^{3}H-DAP with ^{14}C-DIC in the cell wall fragments obtained allows the conclusion that isotope from DIC does indeed enter the cell rather than merely becoming associated with the cell wall.

Effect of reduced glutathione (GSH) on uptake of isotope from DIC-2-^{14}C

In a previous report[8] it was shown that the inhibitory activity of DIC towards *B. subtilis* could be reversed by GSH. This reversal could not be effected by the oxidized form of glutathione (GSSG).* Figure 2(C) indicates that GSH retards the rate of apparent uptake of isotope from DIC-2-^{14}C, presumably in the form of the diazo compound, as well. Since 4-diazoimidazole-5-carboxamide appears to be the active form of DIC in this system, it follows that GSH must either interact directly with the diazo compound or somehow interfere with the decomposition of DIC to form the diazo compound.

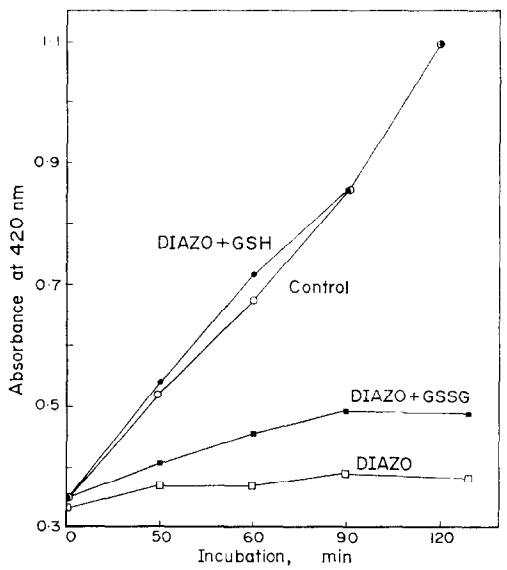

FIG. 4. Effect of GSH on the inhibition of *B. subtilis* growth by 4-diazoimidazole-5-carboxamide. Concentrations were: 4-diazoimidazole-5-carboxamide (DIAZO), 1 μg/ml; GSH, 3 mg/ml; GSSG, 6 mg/ml. Growth curves were obtained as described in Methods.

Interaction of GSH with 4-diazoimidazole-5-carboxamide

If GSH was acting solely by interfering with the breakdown of DIC to the diazo compound, one would not expect it to reverse the action of the diazo compound itself. Figure 4 shows that GSH, but not GSSG, effectively reversed the growth inhibitory activity of 4-diazoimidazole-5-carboxamide towards *B. subtilis*. Neither GSH nor GSSG alone had an effect on the cells.

More direct evidence for an interaction between GSH and 4-diazoimidazole-5-carboxamide in *B. subtilis* growth medium is shown in Fig. 5. When the diazo compound alone was dissolved in minimal medium, it gave an absorption spectrum typical of 2-azahypoxanthine[16] reflecting the rapid cyclization of the compound. When GSH was present, however, a dramatic peak was observed with an absorption maximum around 340 nm. The oxidized form of GSSG did not have this effect.

* P. P. Saunders and G. A. Schultz, unpublished observations.

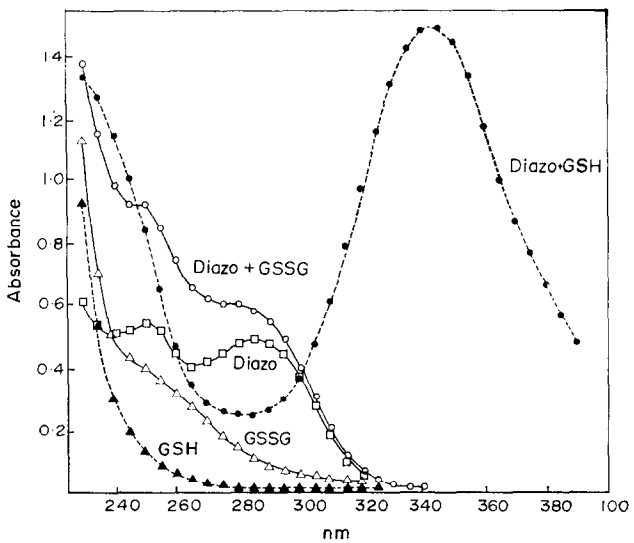

FIG. 5. Effect of GSH on absorption spectrum of 4-diazoimidazole-5-carboxamide. All solutions were made up in minimal medium. Concentrations were: 4-diazoimidazole-5-carboxamide (DIAZO), 20 μg/ml; GSH and GSSG, 1 mg/ml. Readings were made on Zeiss PMQ II spectrophotometer equipped with an automatic sample changer.

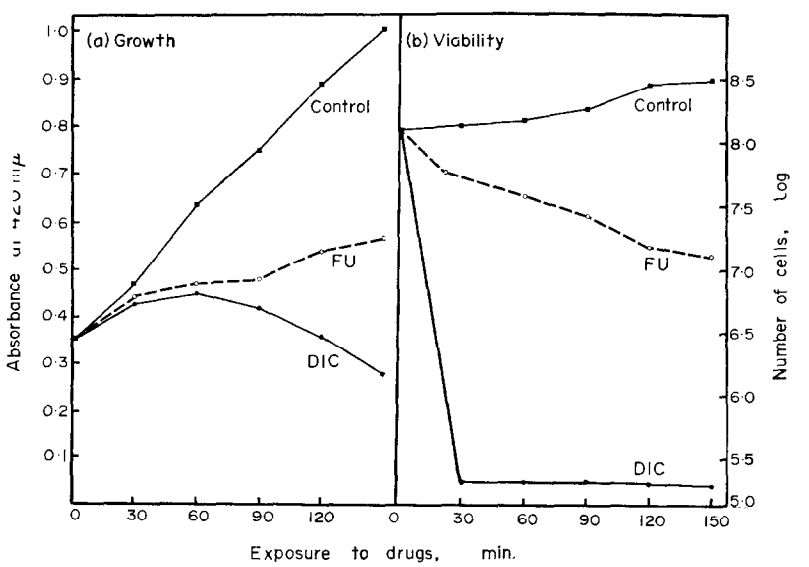

FIG. 6. Effect of DIC on growth and viability of growing *B. subtilis*. Incubation was in the light. The number of viable cells was determined by plating as previously described.[12] The levels of both DIC and FU were 10 μg/ml.

This phenomenon could also be observed by thin-layer chromatography (Table 2). When limiting amounts of GSH were added to 4-diazoimidazole-5-carboxamide prior to application on a thin-layer plate, an extra u.v. absorbing spot of lower R_f value (0·15 to 0·19) appeared. When GSH was present in vast molar excess of the diazo compound, the spot corresponding to the diazo compound, or 2-azahypoxanthine ($R_f = 0·67$), no longer appeared suggesting that the GSH had interacted with, or altered, 4-diazoimidazole-5-carboxamide such that it migrated more slowly.

TABLE 2. EFFECT OF GSH ON THIN-LAYER CHROMATOGRAPHY OF 4-DIAZOIMIDAZOLE-5-CARBOXAMIDE*

Additions to 0·73 μ-mole 4-diazoimidazole-5-carboxamide		Ultraviolet absorbing spots observed after chromatography	
Compound	Micromoles	At $R_f = 0·67$	At $R_f =$ 0·17–0·19
None		+++	
GSH	0·3	++	+
GSH	1·0	+	++
GSH	3·0	±	+++
GSH	10·0		+++
GSSG	15·0	+++	

* A solution of 4-diazoimidazole-5-carboxamide was prepared and immediately dispensed into tubes containing the indicated amounts of glutathione. Five-μl aliquots of each were applied to a thin-layer plate of cellulose powder MN $300F_{254}$ (Brinkman Instruments, Inc.). The plate was developed in a solvent containing propanol–H_2O, 3 : 1. Spots detected with a Mineralight ultraviolet lamp.

These data collectively allow the conclusion that 4-diazoimidazole-5-carboxamide reacts with the sulfhydryl group of GSH to produce a stable product. This explains the ability of GSH to reverse the inhibitory activity of DIC and the diazo compound by preventing their entry into the cell. This phenomenon could have significant biological implications in the therapeutic use of the drug as well.

Effect of DIC on non-proliferating cells. If an agent inhibits by a chemical mechanism such as alkylation, one should be able to demonstrate the lethality of the drug in the absence of growth. To demonstrate this, the effects on viability of DIC and FU (as a control) were compared in proliferating and non-proliferating cells. Figure 6 shows the effects of the two drugs on the growth (absorbance) and viability of growing *B. subtilis*. DIC was lethal within 30 min as shown in graph B, whereas FU gradually decreased the number of viable cells with time. Since this strain of *B. subtilis* requires tryptophan for growth, it was possible to study the effects of DIC and FU on essentially non-growing cells by doing the experiment in a tryptophan-free medium. Figure 7 shows a comparison of growth (A) and the synthesis of RNA (C), DNA (B), and protein (D) in a complete medium and a medium without tryptophan. The absence of tryptophan clearly permits only a slight amount of growth (cell mass) and no proliferation (Figs. 8 and 9). The effects of DIC and FU were observed by addition

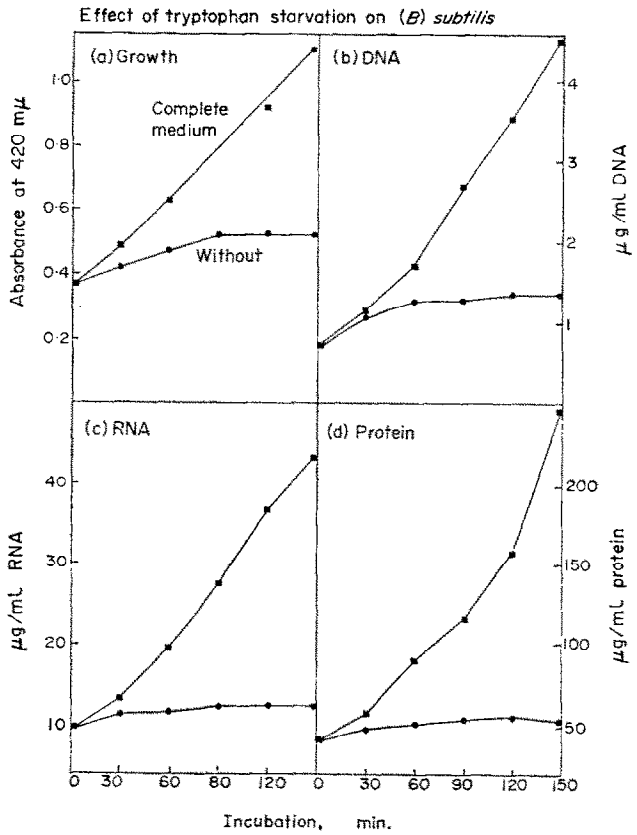

FIG. 7. Growth (a) and the synthesis of DNA (b), RNA (c) and protein (d) in growing (complete medium) and non-growing (medium without tryptophan) *B. subtilis*. Cultures were treated as described in Methods. Levels of DNA, RNA, and protein in aliquots of the cultures were determined colorimetrically as previously described.[12]

of the agents after the cells had incubated for 30 min in the absence of tryptophan to deplete endogenous amino acid. Figure 8 shows that DIC in this situation killed most of the cells during the first 30 min of incubation, an effect quite similar to its effect on actively growing cells (Fig. 6). FU, however, had essentially no effect in the absence of proliferative growth. These data indicate that DIC is indeed active in non-proliferating cells while FU, which requires significant metabolic activity to be inhibitory, is not active. A similar result has been obtained with the diazo compound as shown in Fig. 9. It is of interest to note that 1 μg/ml of the diazo compound showed activity comparable to 10 μg/ml of DIC.

Association in vitro *of DIC-2-^{14}C with proteins.* The experiments of Fig. 10 demonstrate a light-catalyzed association of DIC-2-^{14}C with commercially purified liver alcohol dehydrogenase (ADH) (alcohol-NAD oxidoreductase, EC 1.1.1.1). When this protein was incubated with DIC-2-^{14}C at 37° for 60 min in the light followed by passage through a Sephadex G-50 column, a small peak of radioactivity, all of which was trichloroacetic acid precipitable, appeared in those fractions containing the ADH.

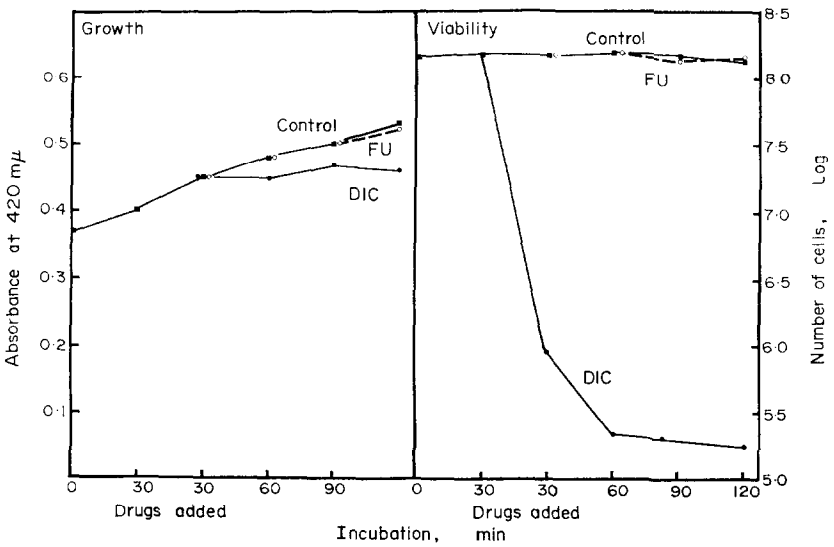

FIG. 8. Effect of DIC on non-proliferating *B. subtilis*. The cells were incubated and treated as described in the absence of tryptophan, a required amino acid. The levels of both DIC and FU were 10 μg/ml.

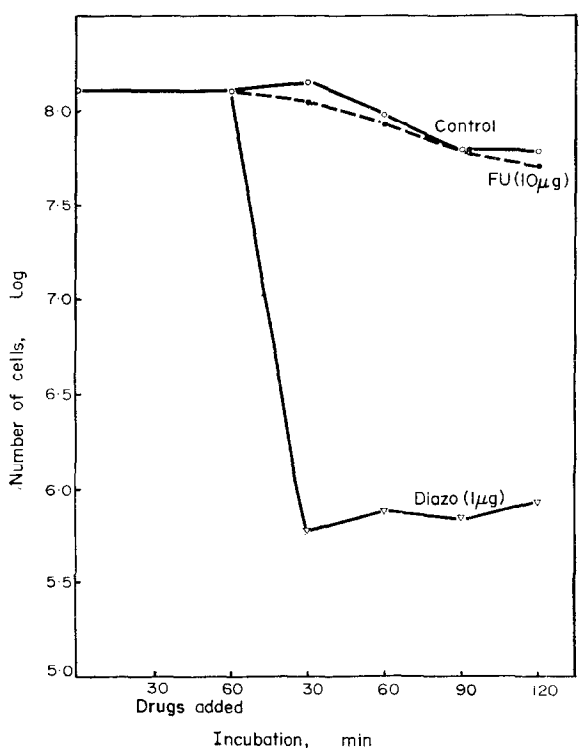

FIG. 9. Effect of 4-diazoimidazole-5-carboxamide on non-proliferating *B. subtilis*. The cells were treated as described in Methods. The levels of drugs were: FU, 10 μg/ml and the diazo compound, 1 μg/ml.

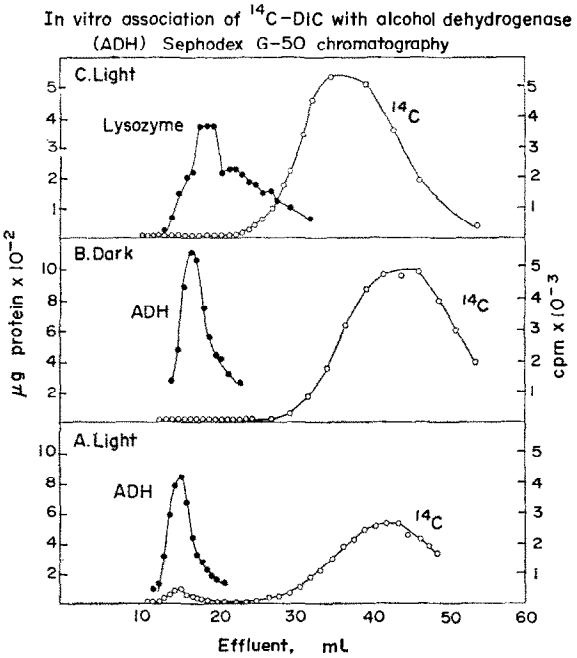

FIG. 10. Sephadex G-50 chromatography of alcohol dehydrogenase (ADH) and lysozyme after incubation with DIC-2-^{14}C. Five mg of each enzyme was incubated with 0·11 μc (2 mg) DIC-2-^{14}C in 0·4 ml 0·05 M Tris-HCl pH 7·5 for 1 hr at 37° in the light (graphs A and C) or in the dark (graph B). Immediately after incubation, the solution was applied to a 1 × 40 cm column of Sephadex G-50, previously equilibrated with 0·05 M Tris-HCl pH 7·5, and elution was done with the same buffer. Fractions of 0·6 to 1·0 ml were collected, and the isotope content was determined by drying an aliquot of each on a Reeve–Angel glass fiber filter (2·4 cm diameter, grade 934 AH) in a counting vial and counting in the toluene base solvent described in Methods. Protein was determined by the method of Lowry *et al.*[17]

When incubation was done in the dark (graph B), there was no isotope associated with the protein peak. Differences in the positions of the peaks from one graph to another reflect tighter packing of the column with use. Since ADH contains several sulfhydryl groups[18] and previous experiments have indicated that the diazo compound interacts readily with sulfhydryl groups, it was of interest to determine whether a light-catalyzed association could be seen between DIC-2-^{14}C and a non-sulfhydryl protein. Graph C shows that no isotope became associated with lysozyme (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17), a protein containing only disulfide groups.[19] These data suggest that a product of DIC, presumably the diazo compound, can interact directly with certain proteins, probably those containing sulfhydryl groups. The relevance of this observation to a primary mechanism of action is questionable; however, it may have some significance in toxicity.

The dependence upon the decomposition of DIC to 4-diazoimidazole-5-carboxamide for inhibition of *B. subtilis* appears to be related, at least in part, to entrance of the drug into the cells. The ability of reduced glutathione to reverse inhibition (and the inability of oxidized glutathione to do so) appears to reflect the ability of the diazo

compound to interact with the sulfhydryl group to form a complex which does not readily enter the cell.

Once inside the cell, the diazo compound may be able to interfere with cellular activities by a number of mechanisms. The fact that it can interact with certain proteins infers that it could inhibit various enzymes or modify structural proteins. Activity of this type would be consistent with a cytotoxic mechanism of action as suggested by the inhibition of non-proliferating cells.

Whether an inhibitory mechanism of this type bears any relevance to the action of DIC which is observed in man has not been resolved. Skibba *et al.*[7] have proposed that, in man, DIC is metabolized to 4(5)-(3-monomethyl-1-triazeno) imidazole-5(4)-carboxamide (MIC) which can spontaneously decompose to yield diazomethane, an alkylating agent, and AIC. This is similar to the proposed mechanism of carcinogenesis by phenyldimethyltriazene proposed by Preussman *et al.*[20] If, however, any decomposition of DIC to the diazo compound occurs as well, it is likely that the latter would contribute to the activity of the drug, the degree being totally dependent upon the amount of photodecomposition that took place. Thus, it is probable that the antitumor activity of DIC observed in man reflects a variety of inhibitory mechanisms.

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