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4 (OR 5)-DIAZOIMIDAZOLE-5 (OR 4)-CARBOXAMIDE AND RELATED TRIAZENOIMIDAZOLES AS ANTIBACTERIAL AGENTS: THEIR EFFECTS ON NUCLEIC ACID METABOLISM OF *ESCHERICHIA COLI* B

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Abstract—4(or 5)-Diazoimidazole-5(or 4)-carboxamide (Diazo-ICA) and dialkyltriazenoimidazoles tested at concentrations of 1 μg/ml and 10 μg/ml, completely inhibit the growth of *Escherichia coli* B without causing cell lysis. With the latter compounds there is a lag period before growth inhibition. After exposing growing cells to 0·1–0·5 μg/ml of Diazo-ICA for 60 min, the number of viable cells was only about 5 per cent of that before exposure. This compound also affects resting cells. The inhibitory effect of Diazo-ICA can be abolished by the addition of cysteine, but 4(or 5)-aminoimidazole-5(or 4)-carboxamide, hypoxanthine, DNA, RNA, protein and leucine have no affect on the inhibition. At relatively high concentration, 2-azahypoxanthine, which is a cyclized product of Diazo-ICA, also inhibits cell growth, but its effect is abolished by the addition of hypoxanthine. Diazo-ICA, at a concentration of 0·05 μg/ml, inhibits the biosynthesis of DNA in the cells, whereas synthesis of RNA is slightly stimulated and synthesis of protein is not affected. In *E. coli* B cells incubated with Diazo-ICA (0·01 μg/ml), the incorporation of thymidine-^{3}H into DNA is severely inhibited. However, the incorporation of uridine-^{3}H into RNA is slightly stimulated and that of leucine-^{3}H into protein is not inhibited. These results suggest that the major action of Diazo-ICA in *E. coli* B is to inhibit DNA synthesis, by interfering with SH-groups in biological systems.

THE IMIDAZOLE ring occurs in several physiologically important compounds such as histidine, histamine and the purines. 4(or 5)-Aminoimidazole-5(or 4)-carboxamide (AICA) promotes the growth of some micro-organisms and may be converted into purines by biological or chemical methods.[1–3]

In previous work in our laboratory, AICA, a precursor in purine biosynthesis, was found to be more rapidly utilized by tumour cells than by the tissues of tumour-bearing animals.[4] This suggested that some derivatives of this compound might act as antagonists of AICA in purine biosynthesis. Accordingly, we synthesized several triazenoimidazoles, some of which have been shown to have antitumour activity with experimental neoplasms.[5, 6] Recently, Hano *et al.*[6] and Shealy *et al.*[7–11] confirmed that the active form of these triazeno compounds for tumour inhibition was 4(or 5)-diazoimidazole-5(or4)-carboxamide(Diazo-ICA). Further, Pershin and Shcherbakova[12] showed that this diazonium salt destroys *Mycobacterium tuberculosis*. However, the mode of action of Diazo-ICA was uncertain. These facts, and the knowledge that some

of the masked compounds of Diazo-ICA are being considered for clinical trial[13–15] prompted a study of the mechanism of the cytostatic or cytocidal effect of this compound in relation to protein and nucleic acid biosynthesis.

The present communication reports the comparative potencies of Diazo-ICA and dialkyltriazenoimidazoles in growth inhibition and the effect of Diazo-ICA on the biosynthesis of nucleic acid and protein of *E. coli* B.

MATERIALS AND METHODS

1. *Compounds used*

Several alkyltriazenoimidazoles and the synthetic intermediate, 4(or 5)-diazoimidazole-5(or 4)-carboxamide (Diazo-ICA) were used for microbial analysis. Diazo-ICA was prepared in our laboratory. 4(or 5)-Dimethyltriazenoimidazole-5(or 4)-carboxamide hydrochloride, 4(or 5)-diethyltriazenoimidazole-5(or 4)-carboxamide hydrochloride, 4(or 5)-dipropyltriazenoimidazole-5(or 4)-carboxamide hydrochloride, 4(or 5)-dibutyltriazenoimidazole-5(or 4)-carboxamide hydrochloride, 4(or 5)-diamyltriazenoimidazole-5(or 4)-carboxamide hydrochloride, and 2-azahypoxanthine were prepared as described by Professor Zenichi Horii, Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Osaka University.[6]

2. *Organism*

Escherichia coli strain B was used for the present study. The growth of the bacterial cells was measured turbidimetrically and the cytocidal effect of the test compounds was investigated by counting the bacterial colonies after 24 hr growth on agar plate.

3. *Medium and conditions of culture*

The cell suspension was prepared by the method of Shiba *et al.*[16] A small amount of the bacterial cell culture was transfered to Simmons' medium containing 0·3% glucose (pH 7·0) and incubated at 37°. This medium contains $MgSO_4 7H_2O$ 0·1 g, KH_2PO_4 1·5 g, $(NH_4)_2HPO_4$ 2·5 g, glutamate 3 g and glucose 3 g per 1000 ml of water. Ten ml of this culture were transfered to 100 ml of fresh medium and incubated again at 37° with shaking for about 3 hr. The culture of cells in the logarithmic phase of growth was then centrifuged and the cells were collected. They were diluted with medium to a final optical density of 0·23–0·25 at 660 mμ (5×10^8 cells/ml).

Experiments were carried out by distributing 9 ml portion of the bacterial suspension containing 5×10^8 cells per ml of Simmons' medium in L-shaped culture tubes. Then 1 ml of solution of the test compound was added to the cell suspension. The cell suspension was incubated at 37° with shaking for the indicated time.

4. *Assay of RNA, DNA and protein*

Assays of nucleic acid and protein were carried out by the following procedures. Nucleic acids were extracted with perchloric acid according to a slight modification of the method of Schneider;[17] the whole culture was mixed with 0·5 ml of 10 N perchloric acid solution and kept in an ice-bath for 20 min. Then the precipitate was extracted by heating the sample with 4 ml of 0·5 N perchloric acid at 90° for 15 min. After centrifugation, the nucleic acids in a 1 ml aliquot of the supernatant were measured, with orcinol reagent for RNA,[18] and with diphenylamine reagent for DNA.[19] The precipitate was dissolved in 4 ml of 1 N sodium hydroxide solution and the protein content of 1 ml of sample was measured with Folin–Ciocalteu reagent.[20]

5. Incorporation experiments

The influences of Diazo-ICA on incorporation of tritiated thymidine, uridine and leucine into DNA, RNA and protein were studied in *E. coli* B by the following technique. Cultures of 1·2 ml of *E. coli* B (5×10^8 cells/ml) with or without Diazo-ICA were exposed to 0·1 μc/1·2 ml of tritiated thymidine, uridine or leucine for 15 min at 37° in L-shaped test tubes. Incubations were terminated by addition of 1 ml of 0·5 N perchloric acid. These test tubes were stood in an ice-bath for 20 min and then the suspension was passed through a millipore-filter. Dry samples containing DNA, RNA and protein were obtained by washing the residue on the filter twice with 5 ml of 0·25 N perchloric acid, twice with 5 ml of 90% (v/v) ethanol and finally with 3 ml of acetone, in this order. The radioactivities of these samples were measured in a gas flow counter.

RESULTS

Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on the growth and viability of E. coli *B*

The cytostatic effect of Diazo-ICA on *E. coli* B cells was studied as described in the experimental section. The results are shown in Fig. 1, from which it appears that Diazo-ICA was able to inhibit cell growth completely at a concentration of about 1 μg/ml. In Diazo-ICA free medium the turbidity increased logarithmically during the first hour. In the medium containing 0·01 μg/ml of this compound, the turbidity increased during the first 50 min and then the increase was suppressed.

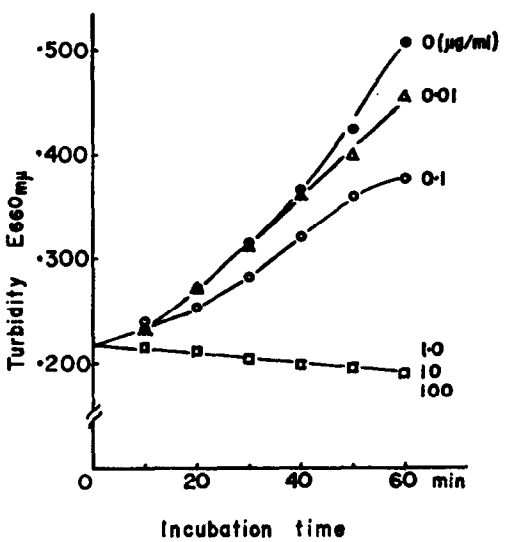

FIG. 1. Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on the growth of *E. coli* strain B.

The number of viable cells in the control was 5×10^8 cells/ml at the start and 2×10^9 after 60 min incubation. A cell count was made on agar plates 24 hr after plating. The increase in viability was parallel to that of turbidity (E_{660}) from 0·09 to 0·900 on incubation for 60 min. As shown in Fig. 2, a dose of 0·1 μg/ml of Diazo-ICA, which did not cause complete growth inhibition, had a significant cytocidal effect. In medium containing Diazo-ICA, the viable cell count was less than 1 per cent of

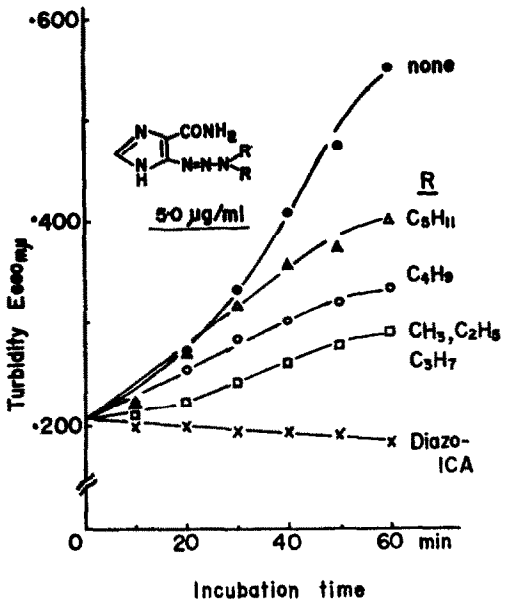

FIG. 2. Effect of 4(or 5)-(dialkyltriazeno)imidazole-5(or 4)-carboxamides on the growth of *E. coli* strain B.

the control after 60 min incubation: 1×10^5 cells/ml with 1 μg/ml and 2×10^4 cells/ml with 10 μg/ml of Diazo-ICA. At a concentration of 0·01 μg/ml of Diazo-ICA, which is the minimum concentration causing inhibition of cell growth, the count of the viable cells decreased to 20 per cent of the control value. This compound affects non-proliferating cells as well as proliferating ones and the results on this are also shown in Table 1.

TABLE 1. VIABLE CELL NUMBER OF *E. coli* STRAIN B IN THE PRESENCE OF 4(OR 5)-DIAZOIMIDAZOLE-5(OR 4)-CARBOXAMIDE

Diazo-ICA (μg/ml)	Incubation time (min)	Simmons' medium	
		Glucose (+)	Glucose (−)
0	0	5×10^8	6×10^8
	60	2×10^9	6×10^8
0·01	0	5×10^8	6×10^8
	60	7×10^8	5×10^8
0·1	0	5×10^8	6×10^8
	60	4×10^8	$3{\cdot}5 \times 10^8$
1	0	5×10^8	6×10^8
	60	1×10^5	3×10^6
10	0	5×10^8	6×10^8
	60	2×10^1	1×10^6
100	0	5×10^8	6×10^8
	60	2×10^3	1×10^3

E. coli B cells in the logarithmic phase of growth were harvested and incubated in Simmons' medium with or without glucose containing various concentrations of the drug with shaking for 60 min at 37°. Then the incubation mixtures were diluted with Simmons' medium and viability of the bacteria was measured on nutrient agar plates by the conventional counting technique.

Comparison of effects of 4(or 5)-(dialklytriazeno)imidazole-5(or 4)-carboxamide on the cell growth of E. coli *B*

Comparative studies on the effects of 4(or 5)-(dialkyltriazeno)imidazole-5(or 4)-carboxamides (Dialkyl-TICAs) on the growth of the bacteria were carried out to elucidate the relation between the length of the alkyl chain and the cytostatic activity of these triazeno derivatives.

4(or 5)-(dimethyltriazeno)imidazole-5(or 4)-carboxamide (Dimethyl-TICA) at a concentration of 5 μg/ml inhibited bacterial growth by approximately 50 per cent relative to that of the control (Fig. 2). In this experiment, the activities of triazeno-imidazole derivatives were compared at equimolar dose levels or a dose of 5 μg/ml of Dimethyl-TICA, as shown in Fig. 3. The dimethyl-, diethyl- and dipropyl- derivatives seemed somewhat more active than the other two compounds.

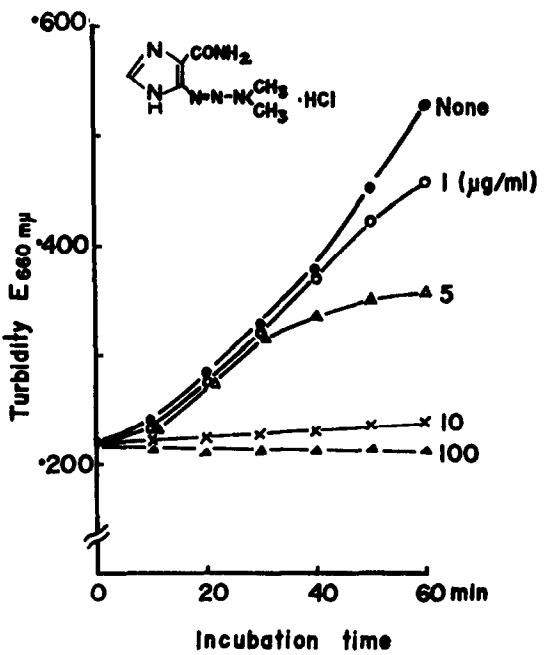

FIG. 3. Effect of 4(or 5)-(dimethyltriazeno)imidazole-5(or 4)-carboxamide on the growth of *E. coli* strain B.

Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on protein and nucleic acid synthesis of E. coli *B*

To elucidate the mechanism of the cytostatic effect of Diazo-ICA, its influences on the protein and nucleic acid contents of *E. coli* were investigated. The results are shown in Table 2 and Fig. 4. In the presence of 0·05 μg/ml of Diazo-ICA (which does not inhibit growth during 60 min incubation), the synthesis of RNA was slightly stimulated in the early stage of incubation, that of protein was not affected at any time during incubation, while DNA biosynthesis was partially inhibited immediately after the addition of the compound. On the contrary, inhibition of RNA and protein synthesis was seen in the presence of 1 μg/ml of Diazo-ICA, a concentration sufficient to prevent the growth of the organism.

TABLE 2. EFFECT OF 4(OR 5)-DIAZOIMIDAZOLE-5(OR 4)-CARBOXAMIDE ON CELLULAR DNA, RNA AND PROTEIN CONTENTS

Diazo-ICA (μg/ml)	DNA-P (μg/10 ml)	RNA-P (μg/10 ml)	Acid sol. RNA-P (μg/10 ml)	Protein (μg/10 ml)
0	8·7	36·0	9·6	1280
0·01	7·8	36·0	9·0	1260
0·1	7·2	37·5	9·6	1190
1	2·4	12·0	6·6	480
10	2·4	9·6	6·0	480
100	1·5	6·0	6·0	480
Time 0	3·3	9·0	4·5	380

Incubation for 60 min

After incubation of *E. coli* B in Simmons' medium with glucose containing various concentrations of the drug for 60 min at 37°, DNA, RNA and protein were extracted by the method described in the experimental section. Acid soluble RNA was assayed using the supernatant obtained by centrifugation the whole culture incubated for 60 min and mixed with 0·5 ml of 10 N perchloric acid and kept in an ice-bath for 20 min.

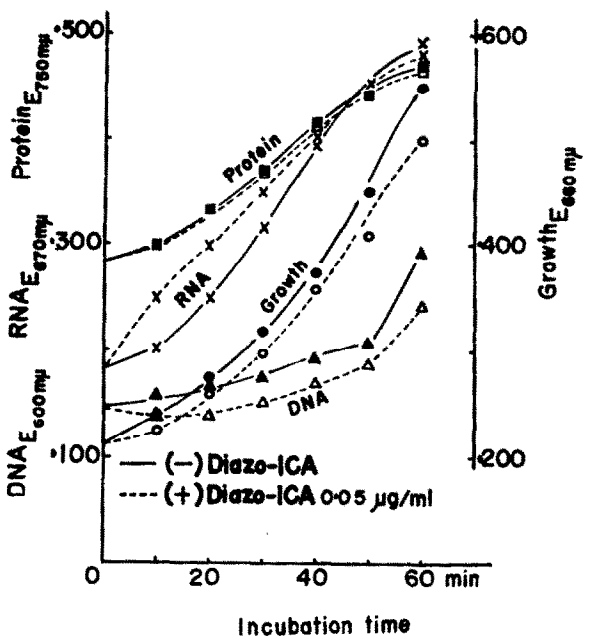

FIG. 4. Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on the growth and DNA, RNA and protein synthesis of *E. coli* strain B.

Antagonistic effects of several compounds on growth inhibition by 4(or 5)-diazoimidazole-5(or 4)-carboxamide

To obtain additional information about the mechanism underlying the action of Diazo-ICA, experiments were made on the prevention of growth inhibition by Diazo-ICA. The various compounds shown in Fig. 5 were added to culture medium containing Diazo-ICA. Diazo-ICA alone, at a concentration of 0·1 μg/ml inhibited

the rate of growth by approximately 50 per cent on incubation for 50 min. The degree of inhibition was not influenced by the presence of DNA (*E. coli* B), RNA (*E. coli* B), protein (bovine albumin), lecithin (egg) or 4(or 5)-aminoimidazole-5(or 4)-carboxamide. The only one of the test compounds which prevented growth inhibition by this diazonium salt was cysteine.

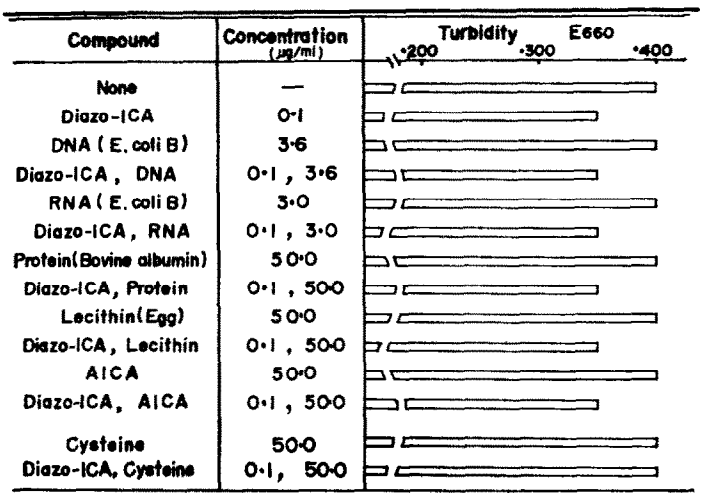

FIG. 5. Effects of various compounds on the growth inhibitory action of 4(or 5)-diazoimidazole-5(or 4)-carboxamide against *E. coli* strain B.

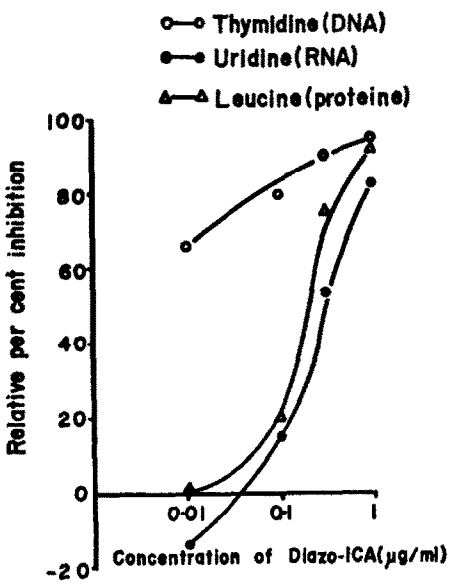

FIG. 6. Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on incorporation of thymidine-^{3}H uridine-^{3}H and leucine-^{3}H into cellular components. The detailed experimental procedures are described in the text. The *E. coli* B cell suspension was incubated for 15 min at 37°.

Effect of 4(or 5)-diazoimidazole-5(or 4)-carboxamide on the incorporation of labelled precursors into DNA, RNA and protein

Figure 6 shows representative data on the influence of Diazo-ICA on the incorporation of thymidine-^{3}H, uridine-^{3}H and leucine-^{3}H into DNA, RNA and protein, respectively, of *E. coli* B. Incubations for incorporation of tritium were made for 15 min at 37°.

At a concentration of 0·01 μg/ml of Diazo-ICA, the incorporation of radioactive precursor into the DNA of the organism was inhibited approximately 65 per cent relative to the control, whereas incorporation of uridine-^{3}H into the RNA fraction was slightly stimulated and that of leucine-^{3}H into protein was not affected at all. Increasing the concentration of this diazonium salt caused more inhibition of incorporation of radioactive thymidine into DNA. Substantial inhibitions of incorporation of radioactive uridine into RNA and leucine into protein were observed with increasing concentrations of Diazo-ICA. The presence of 0·5 μg/ml of Diazo-ICA inhibited incorporation of labelled thymidine, uridine and leucine into DNA, RNA and protein, respectively, by from 60 to 85 per cent.

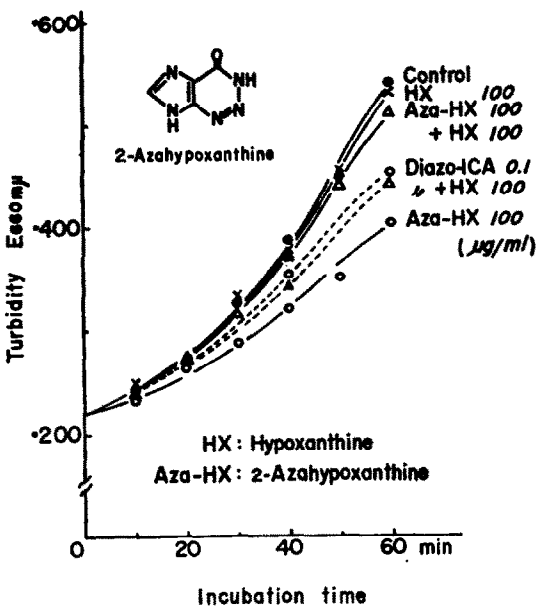

FIG. 7. Effect of hypoxanthine on the growth inhibitory action of 4(or 5)-diazoimidazole-5(or 4)-carboxamide or 2-azahypoxanthine against *E. coli* strain B.

Growth inhibitory activity of 2-azahypoxanthine and its prevention by hypoxanthine

2-Azahypoxanthine which is a cyclized product of Diazo-ICA was shown to have an inhibitory action on bacteria at a relatively high concentration.

2-Azahypoxanthine inhibited bacterial growth by about 30 per cent of the control at a dose of 100 μg/ml, and addition of hypoxanthine at a dose of 100 μg/ml to the 2-azahypoxanthine treated cell culture abolished the inhibitory effect. These results are shown in Fig. 7.

DISCUSSION

The present data show that in the presence of 4(or 5)-diazoimidazole-5(or 4)-carboxamide (Diazo-ICA) at rather low concentration, the growth of *E. coli* B is markedly inhibited. Moreover, the number of viable cells markedly decreased. This may indicate that the growth inhibitory action of Diazo-ICA is not bacteriostatic, but bacteriocidal.

For complete inhibition of bacterial growth, 4(or 5)-(dialkyltriazeno)imidazole-5(or 4)-carboxamide were required at doses of approximately 10 μg/ml, but Diazo-ICA, at a dose of 0·1 μg/ml showed a similar extent of suppression of growth in a culture of *E. coli* B. Moreover, there was a lag period before inhibition was observed with 4(or 5)-(dialkyltriazeno)imidazole-5(or 4)-carboxamides. Therefore, Diazo-ICA might be the active form of the triazeno derivatives for growth inhibition of bacteria. This possibility is supported by the results of experiments on Ehrlich carcinoma.[5] Intraperitoneal injection of Diazo-ICA inhibited Ehrlich ascites carcinoma, but not the solid form of the same tumour. On the other hand, intraperitoneal administration of triazene derivatives with dialkylamines introduced into Diazo-ICA, were active against the solid form of Ehrlich carcinoma, but not against the ascites form.

2-Azahypoxanthine, which is a cyclized form of Diazo-ICA, did not inhibit a transplantable tumour,[6] but the growth of *E. coli* B was partially inhibited by this compound at a concentration of 100 μg/ml. In agreement with these results, Pershin *et al.* have shown that *Mycobacterium tuberculosis* is inhibited by 2-azahypoxanthine and that this inhibition is reversed by hypoxanthine. Further, the antibacterial activity of 2-azahypoxanthine against *E. coli* B is counteracted by hypoxanthine.

In the present experiments, the antagonistic effects on growth inhibition by Diazo-ICA of several compounds, including 4(or 5)-aminoimidazole-5(or 4)-carboxamide, hypoxanthine, DNA and RNA extracted from *E. coli* B and cysteine were evaluated. Cysteine was found to prevent the inhibition.

The antagonistic effect of cysteine was tested, because Iwata *et al.*[21] demonstrated that Diazo-ICA couples with SH-compounds such as cysteine and reduced glutathione and the positive ino- and chronotropic effects of Diazo-ICA on guinea pig atria were reduced or abolished by these SH-compounds.

Preferential inhibition of DNA biosynthesis by Diazo-ICA was most clearly demonstrated at a dose of 0·05 μg/ml and at a higher level, substantial inhibitions of RNA and protein synthesis were observed. The incorporation of thymidine-^{3}H into DNA was inhibited by Diazo-ICA at a concentration of 0·01 μg/ml, whereas the incorporation of uridine-^{3}H into RNA was slightly stimulated rather than inhibited, and incorporation of leucine-^{3}H into protein was not affected. These results and the fact that cysteine completely abolished the Diazo-ICA induced inhibition of the growth of *E. coli* B suggest that the major action of the diazonium salt is to inhibit DNA biosynthesis by interfering with SH-groups in biological systems.

An initial elevation of RNA synthesis and incorporation of labelled uridine into RNA was observed in the presence of a relatively low concentration of Diazo-ICA, whereas DNA synthesis and thymidine incorporation were inhibited at this concentration. Although this finding was not studied further, it is interesting in connection with the mechanism of regulation of bacterial growth.

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