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BIOCHEMICAL STUDIES OF THE EFFECTS OF 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE (IQ-1) IN *ESCHERICHIA COLI* B*

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Abstract—1-Formylisoquinoline thiosemicarbazone (IQ-1) was inhibitory to the growth of *Escherichia coli* B. Growth inhibition was readily reversed either by washing or by the addition of ferrous or ferric ions. The biosynthesis of RNA was the most sensitive to IQ-1 of the metabolic processes measured; DNA synthesis was also inhibited, but was less susceptible, and the formation of protein and cell respiration were unaffected by growth-inhibitory concentrations of IQ-1. The incorporation of ^{14}C-uracil into RNA and DNA was depressed to a greater degree than was the utilization of ^{14}C-adenine for the formation of these macromolecules, suggesting the presence of two sites of action for IQ-1 in this organism, one site resulting in general blockade of RNA synthesis, and the other interferring specifically with the metabolic utilization of uracil. Results of structure–activity relationships suggest that blockade of the biosynthesis of RNA by IQ-1 is at least in part related to the growth-inhibitory potency of this agent. The findings indicate that the mechanism of action of IQ-1 in *E. coli* B is different from that occurring in neoplastic cells, which primarily involves inhibition of DNA synthesis.

A VARIETY of α-(N)-heterocyclic carboxaldehyde thiosemicarbazones have been shown to exhibit: (1) marked antineoplastic activity against a variety of transplanted rodent tumors[1] and spontaneous lymphomas of dogs,[2] (2) weak carcinostatic potency in man,[3,4] and (3) inhibitory activity on DNA viruses of the herpes group.[5] These derivatives are potent inhibitors of ribonucleoside diphosphate reductase from mammalian sources,[5–7] an enzyme which is of central importance to the replication of DNA, catalyzing the reduction of ribonucleotides to deoxyribonucleotide forms. Lesser blockade of the formation of RNA and protein occurs in neoplastic cells treated with α-(N)-heterocyclic carboxaldehyde thiosemicarbazones;[8,9] the molecular mechanisms that account for the drug induced interference with these latter biochemical processes are unknown.

The ribonucleoside diphosphate reductase enzyme of *Escherichia coli*, although similar in mechanism of action to the catalyst derived from mammalian sources, is resistant to the inhibitory action of α-(N)-heterocyclic carboxaldehyde thiosemicarbazones,[6,7] even though the growth of *E. coli* is retarded by these agents.[10] These findings suggest that the biochemical mechanisms by which heterocyclic carboxaldehyde thiosemicarbazones interfere with the growth of *E. coli* are different from the effects responsible for interference with the replication of mammalian cells. The data

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presented in this report using 1-formylisoquinoline thiosemicarbazone (IQ-1), one of the most potent members of the tumor-inhibitory α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones as a model compound, indicate that in the microbial system the biosynthesis of RNA is more sensitive than is the formation of DNA.

MATERIALS AND METHODS

Escherichia coli B was maintained on nutrient agar plates and stored at 4°; plates were reinoculated every 30 days to maintain a fresh culture. For each experiment, an inoculum of *E. coli* was placed in a tube containing 10 ml of C medium with glucose,[11] and incubated for 18 hr at 37°. Cultures were then diluted with fresh C medium containing glucose, and growth was followed by measuring turbidity using a Klett–Summerson colorimeter with either a 420 or 660 nm filter. Measurement of the effects of IQ-1 on the synthesis of cellular macromolecular components was carried out by incubating at 37° a freshly inoculated culture of *E. coli* in C medium until the culture was in the logarithmic phase of growth and contained approx. 3×10^8 cells/ml. The culture was divided into the desired number of pre-warmed flasks which contained the appropriate ^{14}C-labeled precursor, and incubation was continued for various periods of time. ^{14}C-Uracil (25 μmoles; 0·22 μCi/μmole), ^{14}C-adenine (25 μmoles; 0·8 μCi/μmole), ^{14}C-thymidine (66 μmoles; 30 nCi/μmole) and ^{14}C-valine, (20 μmoles; 0·33 μCi μmole) were used to measure the syntheses of RNA, DNA and protein. Incorporation of each labeled precursor was followed for various time periods up to 15 min. The method of Roodyn and Mandel[12] using Millipore filtration was employed for the separation of RNA, DNA and protein constituents of the bacterial cells.

Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer by counting wet filter paper circles (0·45 μ pore size) in either toluene POPOP (4·2 g/l of 2,5-diphenyloxazole and 52·5 mg/l of *p*-bis[2-(5-phenyloxazolyl)]benzene) or ethanol–toluene POPOP (which contained 33% v/v ethanol) for aqueous samples. The fluor was purchased in concentrated solution from New England Nuclear (Liquifluor).

IQ-1 was obtained from several sources: the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.; a donation from Mr. Frederic A. French; and some was synthesized in our laboratory. All chemical derivatives used for the structural modification studies were also synthesized in our laboratory. IQ-1 was dissolved in dimethylsulfoxide and diluted 1:100 with the assay mixture or bacterial culture depending upon the experiment; 0·5% dimethylsulfoxide was the lowest concentration that kept IQ-1 in solution throughout the experiments employed and was not inhibitory to growth of *E. coli* or the metabolic processes measured under the conditions of these experiments.

RESULTS

The effects of varying concentrations of IQ-1 on the growth of *E. coli* B were measured. Increasing the level of inhibitor resulted in an increase in the degree of inhibition: a concentration of $2{\cdot}5 \times 10^{-5}$ M IQ-1 was calculated to be the approx. 50 per cent growth-inhibitory level. Phase contrast and electron microscopic studies did not demonstrate any discernible difference between untreated cells and those exposed to concentrations of IQ-1 that caused pronounced inhibition of growth.

The reversibility of the inhibition by IQ-1 on the growth of *E. coli* B was tested by removal of inhibitor from the growth medium; the findings are illustrated in Fig. 1. Two concentrations of IQ-1 were used to inhibit growth, $2{\cdot}5 \times 10^{-5}$ and 10^{-4} M, and initial growth rates were established over a period of 90 min. At this time, cells were collected by centrifugation, then washed and resuspended. Cells treated with

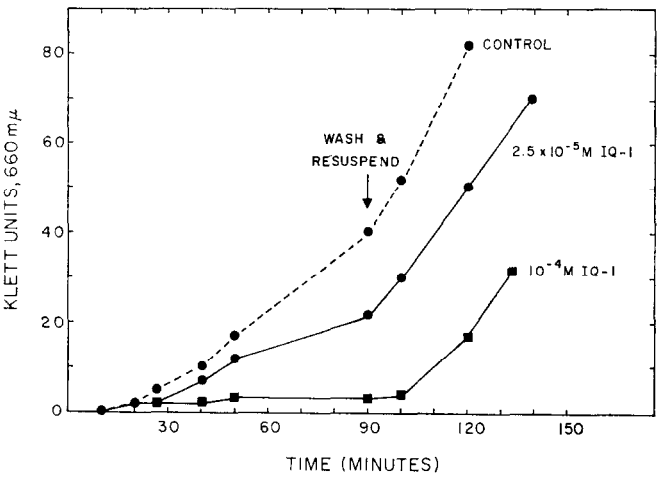


FIG. 1. Reversibility of the inhibition of growth of *E. coli* B by IQ-1 by removal of inhibitor. Bacteria were grown from an 18-hr overnight culture in flasks (15 ml/flask) containing either IQ-1 ($2{\cdot}5 \times 10^{-5}$ or 10^{-4} M) or no drug. After initial growth rates were established for a period of 90 min, cells were collected by centrifugation, washed with 1 ml of warm C medium containing glucose, and resuspended in fresh warm C medium containing glucose. The flasks were reincubated at 37° with shaking, and growth was followed using a Klett–Summerson colorimeter.

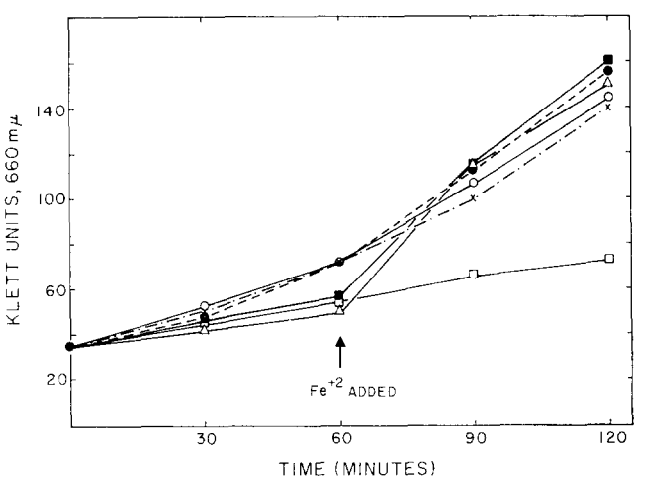


FIG. 2. Effect of ferrous ion on the inhibition of growth of *E. coli* B by IQ-1. Cells were propagated and growth was measured by techniques described in Fig. 1. Ferrous ion was added to the indicated flasks after 60 min of incubation. No drug, no Fe^{2+}, ●– – –●; $2{\cdot}5 \times 10^{-5}$ M IQ-1, no Fe^{2+}, □——□; $2{\cdot}5 \times 10^{-5}$ M IQ-1, 10 μM Fe^{2+}, ■——■; no drug, 10 μM Fe^{2+} ×–·–×; $2{\cdot}5 \times 10^{-5}$ M IQ-1, 100 μM Fe^{2+}, △——△; no drug, 100 μM Fe^{2+}, ○——○.

$2{\cdot}5 \times 10^{-5}$ M IQ-1 re-established a normal growth rate immediately and those exposed to 10^{-4} M IQ-1 re-established a normal growth rate after a relatively short delay.

Previous findings[13,14] have shown that IQ-1 is a relatively strong chelating agent for the transition elements, iron, cobalt, nickel, copper, zinc and manganese. To ascertain the relationship between chelating potential and the inhibition of the growth of *E. coli* by IQ-1, metals were added to cells after 60 min of incubation in the presence of a 50 per cent growth–inhibitory concentration ($2{\cdot}5 \times 10^{-5}$ M) of IQ-1. The findings

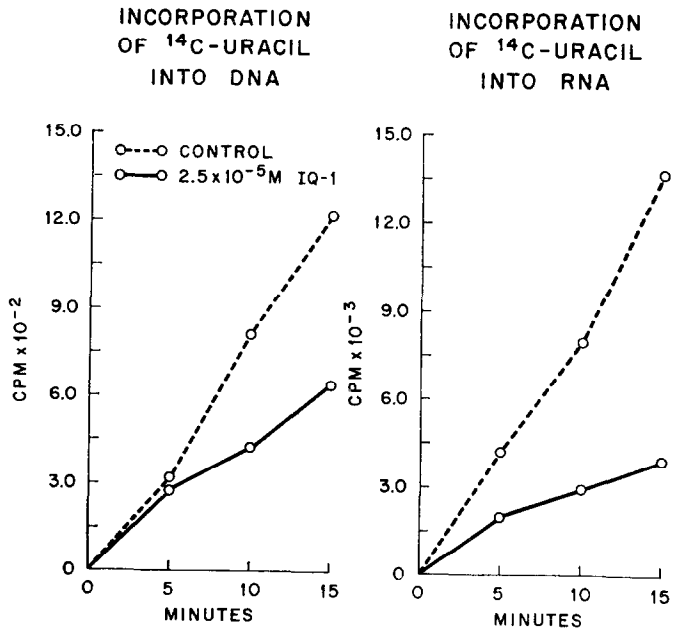


FIG. 3. Effect of IQ-1 on the synthesis of DNA and RNA as measured by the incorporation of ^{14}C-uracil. *E. coli* B were grown from an 18-hr overnight culture into the logarithmic phase of growth and divided into two portions; 25 μmoles ^{14}C-uracil (specific activity, 0·22 μCi/μmole) were added to each flask. One flask contained $2{\cdot}5 \times 10^{-5}$ M IQ-1 solubilized in 0·5% dimethylsulfoxide and the other flask served as a control. Dimethylsulfoxide (0·5%) had no effect on the rate of incorporation of ^{14}C-uracil. The final volume in each flask was 33 ml. Flasks were incubated with shaking at 37° and 10-ml samples were withdrawn 5, 10 and 15 min after addition of the labeled uracil, and radioactivity in the DNA and RNA fractions was measured. The ordinate represents counts/min per 2 ml of culture. Control, O– – –O; $2{\cdot}5 \times 10^{-5}$ M IQ-1, O——O.

obtained with ferrous ion are shown in Fig. 2. They indicate that *E. coli* inhibited by IQ-1 rapidly establish a normal growth after the addition of either 10 or 100 μM Fe^{2+}. These concentrations of Fe^{2+} were neither inhibitory nor stimulatory to *E. coli* grown in the absence of the thiosemicarbazone. Furthermore, the iron chelate of IQ-1 at $2{\cdot}5 \times 10^{-5}$ M had essentially no effect on the growth rate of this organism. To a lesser extent, Fe^{3+} was capable of reversing the growth inhibition of *E. coli* produced by IQ-1, whereas other metals (Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}) that similarly interact with IQ-1 to form co-ordination compounds lacked the ability to reverse growth inhibition.

The biochemical basis for the growth-inhibitory activity of IQ-1 on *E. coli* was investigated. Initial experiments measured the effects of IQ-1 on the respiration of intact cells using a Gilson differential respirometer; the results showed that with concentrations of drug up to 10^{-4} M, a level that stops bacterial growth almost immediately, the cells continued to respire at a relatively active rate. Thus, it appears that IQ-1 does not exert its bacteriostatic activity by interfering with cellular respiratory functions.

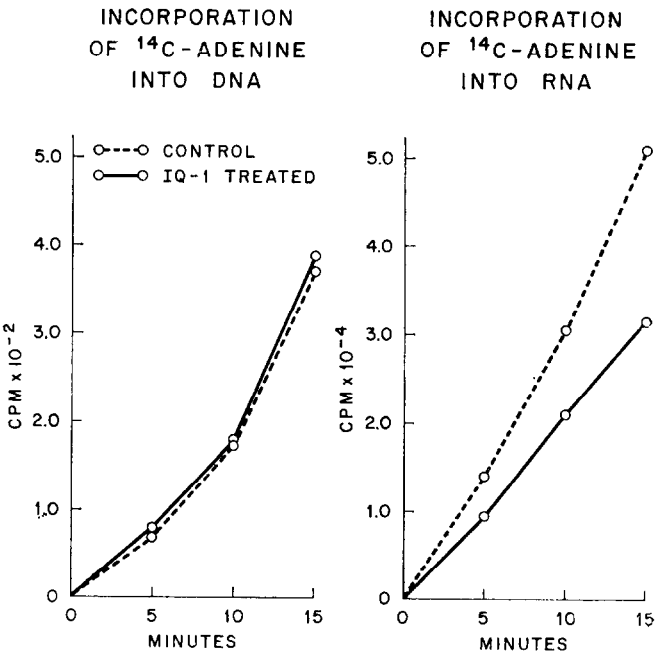


FIG. 4. Effect of IQ-1 on the synthesis of DNA and RNA as measured by the incorporation of ^{14}C adenine. The experimental details were as described in Fig. 3, except that 25 μmoles ^{14}C-adenine (specific activity, 0·8 μCi/μmole) were added to each flask.

The action of the heterocyclic carboxaldehyde thiosemicarbazone on nucleic acid biosynthesis was measured to determine whether this agent created lesions in polynucleotide metabolism; the effect of $2{\cdot}5 \times 10^{-5}$ M IQ-1 on the incorporation of ^{14}C-uracil into RNA and DNA is shown in Fig. 3. The utilization of radioactive uracil for the synthesis of RNA was considerably more sensitive to the metabolic action of IQ-1 than was the incorporation of the labeled pyrimidine into DNA. Thus, after 5 min of incubation in the presence of drug, the formation of RNA was depressed about 50 per cent whereas the biosynthesis of DNA was not affected. Separation of extracted RNA by centrifugation through a 4–20% sucrose gradient indicated that uracil incorporaton into all classes of RNA of *E. coli* was equally inhibited by IQ-1.

The biosynthesis of polynucleotides was also monitored using ^{14}C-adenine, a precursor of the formation of the purine nucleotide moiety of the nucleic acids. The findings (Fig. 4) indicate that the formation of RNA, as monitored with this labeled

precursor, was more sensitive to the inhibitory action of IQ-1 than was DNA, which was not affected by the action of this agent. Comparison of the magnitude of the effects of IQ-1 on nucleic acid biosynthesis using ^{14}C-uracil and ^{14}C-adenine as measures of these processes (Figs. 3 and 4) indicates that the utilization of uracil for nucleic acid formation was considerably more susceptible to the action of the chelating agent than was the incorporation of ^{14}C-adenine.

The relative insensitivity of the biosynthesis of DNA was also demonstrated using ^{14}C-thymidine as a measure of the rate of formation of DNA. A concentration of $2{\cdot}5 \times 10^{-5}$ M IQ-1, a 50 per cent growth-inhibitory level, caused only slight de-

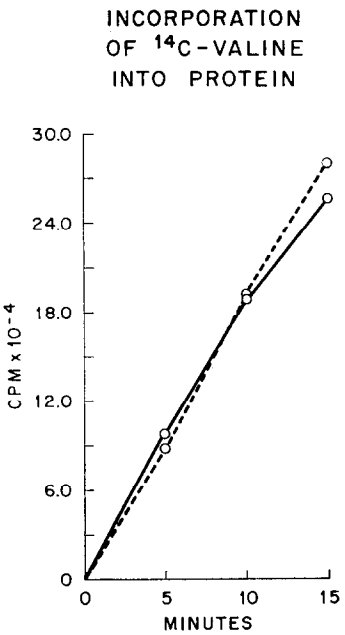


FIG. 5. Effect of IQ-1 on the incorporation of ^{14}C-valine into protein. The experimental details were as described in Fig. 3, except that 20 μmoles ^{14}C-valine (specific activity, 0·33 μCi/μmole) were added to each flask.

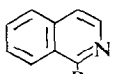
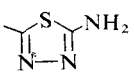
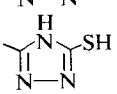
pression of the utilization of thymidine. Two times the 50 per cent growth-inhibitory concentration of the drug, however, caused a marked depression of the incorporation of ^{14}C-thymidine into DNA.

To gain further evidence for specificity of action of the metabolic inhibitor, the effect of IQ-1 ($2{\cdot}5 \times 10^{-5}$ M) on the synthesis of protein was measured. The results shown in Fig. 5 indicate that a cytostatic concentration of this agent did not alter the rate of incorporation of ^{14}C-valine into the protein of *E. coli*.

Studies in mammalian cells have defined in part the structural features required for both the growth-inhibitory activity of IQ-1[15,16] and its ability to inhibit the enzyme ribonucleoside diphosphate reductase and consequently DNA synthesis.[6,7,17] To gain similar information as to the structural parameters responsible for the action of

IQ-1 on both the synthesis of RNA and growth in *E. coli*, the effects of various structural modifications were determined and the results are shown in Tables 1 and 2. In general, a close correlation existed between the ability of a compound to inhibit cellular growth and its corresponding potency as an inhibitor of the synthesis of RNA. Modification of the formyl thiosemicarbazone (—CH=NNHCSNH$_2$) side chain of

TABLE 1. EFFECT OF MODIFICATION OF THE THIOSEMICARBAZONE SIDE CHAIN ON THE GROWTH OF *E. coli* AND THE SYNTHESIS OF RNA

 Compound (R)	% Inhibition	
	Growth*	RNA synthesis†
—CH=NNHCSNH$_2$‡	68	87
—C(CH$_3$)=NNHCSNH$_2$	30	10
—CONHNHCSNH$_2$	48	77
—CH=NN(CH$_3$)CSNH$_2$	0	6
—CH=NN(C$_6$H$_5$)CSNH$_2$	0	0
—CH=NNHC(=NH)NH$_2$	27	0
—CH=NNHCONH$_2$	12	5
—CH=NN=C(SCH$_3$)NH$_2$	22	11
—CH=NN=C(SO$_3$H)NH$_2$	9	5
—CH=NN=C(SCOCH$_3$)NHCOCH$_3$	29	0
—CH=NNHCS$_2$Na	29	4
—CH=NNHCSNHNHCH$_3$	75	98
—CH=NNHCS$_2$CH$_3$	31	16
—CH=NNHCSNHCH$_2$CH$_2$OH	53	82
—CH=NNHCSNHCH$_2$CH(CH$_3$)$_2$	44	45
—CH=NNHCSN⟨ ⟩O	100	99
	22	10
	9	15

* Bacterial growth inhibition was measured after 2 hr of incubation in the presence of $2{\cdot}5 \times 10^{-5}$ M drug. Results are indicative of a representative experiment.

† Inhibition of RNA synthesis was determined by incorporation of ^{14}C-uracil into bacterial RNA, as described in Fig. 4.

‡ 1-Formylisoquinoline thiosemicarbazone (IQ-1).

IQ-1[15] led in most instances to a decrease in inhibitory activity; replacement of the terminal amide group with either a methylhydrazino moiety or a morpholino ring, however, resulted in compounds having greater activity than IQ-1. Attachment of the formyl thiosemicarbazone side chain of IQ-1 to other ring systems such as pyrazine, pyridine, purine, or benzene markedly lowered inhibitory potency (Table 2).

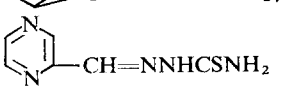
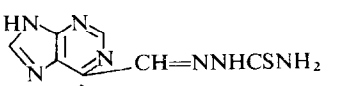
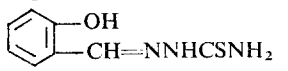
DISCUSSION

Inhibition of the biosynthesis of DNA by IQ-1, which occurs as a result of blockade of the enzyme ribonucleoside diphosphate reductase, has been demonstrated, through studies of structure–activity relationships, to be essential for interference with

the replication of transplanted neoplastic cells.[6,7,17–19] Such a target site was not responsible for the growth-inhibitory activity of IQ-1 in *E. coli* B, since the ribonucleoside diphosphate reductase enzyme of this organism was found to be insensitive to the action of this agent.[6] Thus, it appeared that the biochemical mechanism responsible for the cytotoxicity of the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones was different in the mammalian and bacterial systems.

The finding that washing of *E. coli* cells completely inhibited by IQ-1 resulted in restoration of a normal growth rate indicated that the action of this agent was reversible; this contrasts with the finding that the action of IQ-1 is not reversible in

TABLE 2. EFFECT OF MODIFICATION OF THE HETEROCYCLIC RING SYSTEM ON THE GROWTH OF *E. coli* AND THE SYNTHESIS OF RNA

Compound	% Inhibition	
	Growth*	RNA synthesis†
	68	87
	37	22
	55	64
	20	7
	7	7

* Bacterial growth inhibition was measured after 2 hr of incubation in the presence of $2{\cdot}5 \times 10^{-5}$ M drug. Results are indicative of a representative experiment.

† Inhibition of RNA synthesis was determined by incorporation of ^{14}C-uracil into bacterial RNA, as described in Fig. 4.

‡ 1-Formylisoquinoline thiosemicarbazone (IQ-1).

neoplastic cells and thereby produces long-term survival of mice bearing tumor transplants.[20,21] Normal growth of *E. coli* was reinstated in inhibited cultures by the addition of either ferrous or ferric ion. Other metals, such as zinc, cobalt, copper and nickel, which form co-ordination complexes with IQ-1, as do ferrous and ferric ions,[13,14] were unable to reverse the growth-inhibitory activity of IQ-1. These results suggest that either: (1) iron, because of its greater affinity for the inhibitor than the other metals employed, effectively removes it from the target site(s), or (2) IQ-1 interferes with a critical enzymatic reaction(s) that relies on trace quantities of ferrous or ferric ions.

Studies with a variety of radioactive precursors of the syntheses of nucleic acids and protein have indicated that the biosynthesis of RNA is both the most sensitive process to the action of IQ-1 and the one that is initially affected by this agent. The formation of DNA is considerably less inhibited by the heterocyclic carboxaldehyde thiosemicarbazone, and protein synthesis and respiration are relatively resistant to the action of

this compound. The finding that the incorporation of adenine into both RNA and DNA is considerably less susceptible to the inhibitory effects of IQ-1 than is the utilization of uracil for the formation of these macromolecules suggests the presence of at least two sites of action, one that results in general blockade of RNA synthesis and is monitored with both ^{14}C-adenine and ^{14}C-uracil, and the other on the pathways involved in the metabolism of uracil. Such a mechanism was substantiated by the findings, reported in the accompanying manuscript,[22] that IQ-1 interfered with the activities of both pyrimidine nucleoside monophosphate kinase and RNA polymerase of *E. coli*.

That inhibition of the biosynthesis of RNA by IQ-1 is related, at least in part, to the bacteriostatic activity of this agent was suggested by comparison of the effects of various structural modifications of IQ-1 on both the multiplication of *E. coli* and the biosynthesis of RNA in this organism. Relatively good correlation existed between the capacity of these derivatives to interfere with growth and their abilities to affect the formation of RNA.

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