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LOCALIZATION IN *ESCHERICHIA COLI* B OF TWO ENZYMATIC SITES OF ACTION BY 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE (IQ-1) ON RIBONUCLEIC ACID BIOSYNTHETIC PATHWAYS*

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Abstract—The site(s) of action of 1-formylisoquinoline thiosemicarbazone (IQ-1) on RNA biosynthetic pathways was investigated in *Escherichia coli* B by measuring the effects of this drug on the conversion of ^{3}H-uridine and ^{3}H-adenosine to acid-soluble nucleotide forms. The incorporation of ^{3}H-uridine into acid-soluble UMP and CMP was unaffected by a 50 per cent growth-inhibitory concentration of IQ-1; however, the progression of radioactivity from pyrimidine nucleoside monophosphates to di- and triphosphates was markedly depressed. In contrast, the conversion of ^{3}H-adenosine to purine nucleotide forms was unaffected by IQ-1. A partially purified preparation of ATP: nucleoside monophosphate phosphotransferase from *E. coli* was used to show that pyrimidine nucleoside monophosphate kinase activity was sensitive to IQ-1. A second locus of action appeared to be RNA polymerase; variable inhibition, which seemed to depend upon the degree of purity of the enzyme, was produced by IQ-1. Several structurally related compounds were tested for potency against pyrimidine nucleoside monophosphate kinase and RNA polymerase; the results showed close correlation between the potential for inhibition of growth and the degree of interference with the activity of these enzymes, suggesting the involvement of these lesions in the bacteriostatic action of IQ-1.

TREATMENT of neoplastic cells with 1-formylisoquinoline thiosemicarbazone (IQ-1) results in a pronounced suppression of the biosynthesis of DNA, coupled with a lesser degree of interference with the formation of RNA and protein.[1–5] In the bacterial system *E. coli*, however, the primary site of cytostatic action, as determined by metabolic studies and structure–activity relationships, appears to be on the RNA biosynthetic pathways.[6,7] The findings attained with the use of radioactive precursors of the nucleic acids[7] suggest the presence of two drug-sensitive loci, one on the metabolic reactions involved in the metabolism of uracil nucleotides, and the other, the cause of a more general depression of the formation of RNA.

The present investigation was designed to localize the sites of action of IQ-1 on the RNA synthetic pathways. The results obtained indicate that, in *E. coli*, α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones interfere with the synthesis of RNA by inhibition of the activities of the enzymes, pyrimidine nucleoside monophosphate kinase and RNA polymerase.

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MATERIALS AND METHODS

E. coli B was grown in C medium containing glucose for 18 hr at 37°; cultures were then diluted with fresh C medium with glucose, and growth was measured using a Klett–Summerson colorimeter as described previously.[7]

To determine the effects of IQ-1 on the synthetic pathways involved in the synthesis of RNA, overnight cultures (18 hr) of *E. coli* B were used to establish logarithmic phase growing cultures. When the cell number per millimeter was approx. 3×10^8, the cultures were divided into three flasks: an untreated control, a dimethyl sulfoxide control, and a flask to which $2{\cdot}5 \times 10^{-5}$ M IQ-1 was added. ^{3}H-Uridine (7·5 mCi/μmole) was used to investigate the effects of IQ-1 on pyrimidine nucleotide biosynthesis and ^{3}H-adenosine (8 μCi/μmole) to monitor the action of the drug on the purine nucleotide pathways. After 10 min of incubation at 37° with shaking, an equal volume of cold 8% perchloric acid (PCA) was added, and the flasks were immediately placed in a salt water–ice bath. The rate of incorporation of these labeled precursors into the nucleic acids was determined by the method of Roodyn and Mandel.[8] The acid-soluble fraction was collected, neutralized with KOH and $KClO_4$ was removed by centrifugation. An aliquot (usually 5 or 10 ml) of this neutralized acid-soluble fraction was mixed with appropriate carrier nucleotides and applied to a Dowex-1-formate column (1·5 × 15 cm). The column was washed with 50 ml of distilled water, and elution of nucleotides was carried out using a linear gradient of ammonium formate from 0 to 1·6 M.[9] The total gradient was 1000 ml and the flow rate was approx. 0·5 ml/min or less. The nucleotide content of the fractions was measured at 260 nm and aliquots (0·5 ml) of each sample (5–10 ml) were monitored for radioactivity with a Packard Tri-Carb liquid scintillation spectrometer. Ultraviolet spectra were obtained from each peak to verify its identity.

RNA polymerase was purified either according to the method of Burgess[10] or by a modification of the technique of Chamberlain and Berg.[11] The modified Chamberlain–Berg preparation employed frozen ¾ logarithmic phase *E. coli* B cells obtained from Grain Processing Co. Sixty g of frozen *E. coli* B were thawed overnight at 4° in 100 ml of 0·01 M Tris–HCl containing 0·01 M magnesium acetate and 10^{-4} M EDTA and ground with washed glass beads for 15 min in a Waring blender. To maintain the temperature below 5°, beads were precooled to −70°, and the grinding was done at 4°. The supernatant fluid was centrifuged at 30,000 *g* for 30 min, the supernatant was made 0·01 M with respect to β-mercaptoethanol, and the pH was adjusted to 8·0 using 1 M Tris–HCl. This solution was centrifuged for 2 hr at 100,000 *g*. The supernatant fluid was treated with 0·13 ml of a freshly prepared 10% solution of streptomycin sulfate per milliliter of supernatant fluid. After 15 min, the resulting precipitate was centrifuged at 30,000 *g* for 30 min. The supernatant fluid was then treated with a 1% suspension of protamine sulfate in an amount such that 90 per cent of the polymerase activity was precipitated. The pellet was resuspended in 0·01 M Tris–HCl, pH 7·9, containing 0·01 M magnesium acetate, 10^{-4} M EDTA, and 0·01 M β-mercaptoethanol. After centrifugation at 30,000 *g* for 30 min, the pellet was extracted in a similar manner using 0·01 M Tris–HCl, pH 7·9, containing 0·01 M magnesium acetate, 10^{-4} M EDTA, 0·1 M $(NH_4)_2SO_4$ and 0·01 M β-mercaptoethanol. The suspension was centrifuged at 30,000 *g* for 30 min and the supernatant fluid collected. The supernatant fluid was made 35 per cent saturated with respect to $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 30,000 *g* for 30 min and was resuspended in a

storage buffer containing 0·01 M Tris–HCl, pH 7·9, 0·01 M $MgCl_2$, 0·1 M KCl, 0·1 mM dithiothreitol, 0·1 mM EDTA and 50% glycerol, so that the final protein concentration exceeded 5 mg/ml. The solution was stored at −20°.

RNA polymerase activity was assayed using a mixture containing 0·04 M Tris–HCl (pH 7·9 at 25°), 0·1 M dithiothreitol, 0·15 M KCl, 0·01 M $MgCl_2$, 0·1 mM EDTA, 150 μM each of UTP, GTP, CTP and ^{14}C-ATP (1 mCi/mmole), and 0·15 mg/ml of calf thymus DNA in a final volume of 0·25 ml. The mixture was incubated at 37° for 10 min at which time an equal volume of 10% ice-cold TCA was added, and the tubes were placed in ice for a period of 15 min. The precipitate was collected on Millipore filters (0·45 μ), the filter discs were washed three times with 1% TCA which contained 0·01 M sodium pyrophosphate and the radioactivity present in the disc was determined.

ATP : nucleotide phosphotransferase (nucleotide kinase) was prepared as described by Canellakis *et al.*[12] The assay mixture used to measure nucleotide kinase activity contained: $MgCl_2$, 6 μmoles; ATP, 0·6 μmole; phosphocreatine, 7 μmoles; creatine kinase, 40 μg; Tris–HCl, pH 7·4, 20 μmoles; nucleoside monophosphate, 2 μmoles; and 0·1 ml of *E. coli* B enzyme in a final volume of 0·5 ml. Incubation was carried out at 37° for varying periods of time up to 30 min. An equal volume of cold 8% PCA was added to terminate the reaction and the mixture was placed in ice for 10 min. The suspension was filtered through 0·45 μ Millipore filters, and each filter disc was washed three times with 1 ml of 1% PCA. The acid-soluble fraction was collected and neutralized to pH 7 using KOH. The volume was recorded, and an aliquot of usually 20 μl was applied to MN-Polygram PEI-Cel 300 thin-layer sheets (Brinkman Instrumentation Company). The PEI-cellulose sheets were developed for 1 hr in 1 M LiCl to separate nucleoside mono-, di- and triphosphates.[13] Nucleotides were located with ultraviolet light and areas corresponding to the various nucleotides were cut out, placed in vials containing 20 ml of scintillation mixture (4·2 g of 2,5-diphenyloxazole and 52·5 mg of *p*-bis-(5-phenyloxazol-2-yl)-benzene/liter of toluene), and radioactivity was determined.

RESULTS

To localize the action of IQ-1 on the biosynthetic pathways involved in the conversion of uracil nucleotides to the nucleic acids, the effects of a 50 per cent growth-inhibitory concentration ($2·5 \times 10^{-5}$ M) of the heterocyclic carboxaldehyde thio-

TABLE 1. EFFECT OF IQ-1 ON THE INCORPORATION OF ^{3}H-URIDINE AND ^{3}H-ADENOSINE INTO ACID-PRECIPITABLE MATERIAL*

Precursor	IQ-1 treatment	Radioactivity (Counts/min/10 ml of culture $\times 10^{-3}$)
^{3}H-uridine	−	320
	+	95
^{3}H-adenosine	−	1030
	+	840

* Logarithmic phase *E. coli* cells were grown in the presence and absence of IQ-1 ($2·5 \times 10^{-5}$ M) as described in Fig. 1.

semicarbazone on the conversion of ^{3}H-uridine to acid-soluble nucleotide forms were measured in logarithmic growth cultures of *E. coli*. The findings using a 10-min pulse of ^{3}H-uridine are shown in Fig. 1, A and B. Treatment with IQ-1 did not affect the utilization of uridine for the synthesis of pyrimidine nucleoside monophosphates, but

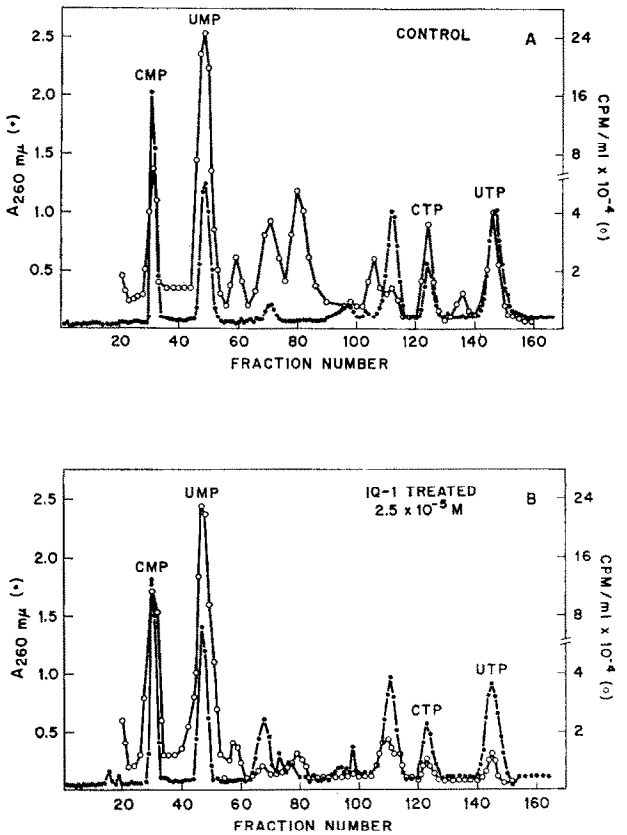


FIG. 1. Effect of IQ-1 on the incorporation of ^{3}H-uridine into acid-soluble pyrimidine nucleotides of *E. coli* B. Logarithmic phase *E. coli* cells were divided into three flasks: an untreated control, a 1% DMSO control, and a treated culture (IQ-1, $2{\cdot}5 \times 10^{-5}$ M). ^{3}H-Uridine (7·5 mCi/μmole) was added to a final concentration of 13·2 μM. After 10 min of incubation with shaking, growth was terminated by addition of cold 8% PCA. Nucleic acids were separated by Millipore filtration from the acid-soluble fraction, and separation of nucleotides from the soluble fraction was accomplished using Dowex-1-formate columns eluted with an ammonium formate gradient as described in Materials and Methods. Nucleotides were identified by their ultraviolet spectral properties. The open circles (○——○) represent radioactivity and the closed circles (●——●) absorbancy. A, untreated control; B, IQ-1-treated

decreased markedly the incorporation of radioactivity from ^{3}H-uridine into cytosine and uracil nucleotide di- and triphosphates. This resulted in a pronounced decrease in the labeling of the nucleic acids from uridine in cells treated with the chelating agent (Table 1). Since considerably less depression of the synthesis of RNA by IQ-1 occurred when precursors of the purine nucleotide pathways were used as the metabolic tracers[7] (Table 1), an identical experiment was conducted to ascertain the effects of IQ-1 on

the conversion of ^{3}H-adenosine to acid-soluble nucleotide forms (Fig. 2, A and B). No significant difference was observed between untreated cells and those exposed to IQ-1 on the incorporation of adenosine into purine nucleotides.

The finding that ^{3}H-uridine incorporation into pyrimidine monophosphates was not affected by IQ-1, while the subsequent passage of radioactivity into higher nucleotide forms was markedly depressed, suggested inhibition of the enzyme pyrimidine

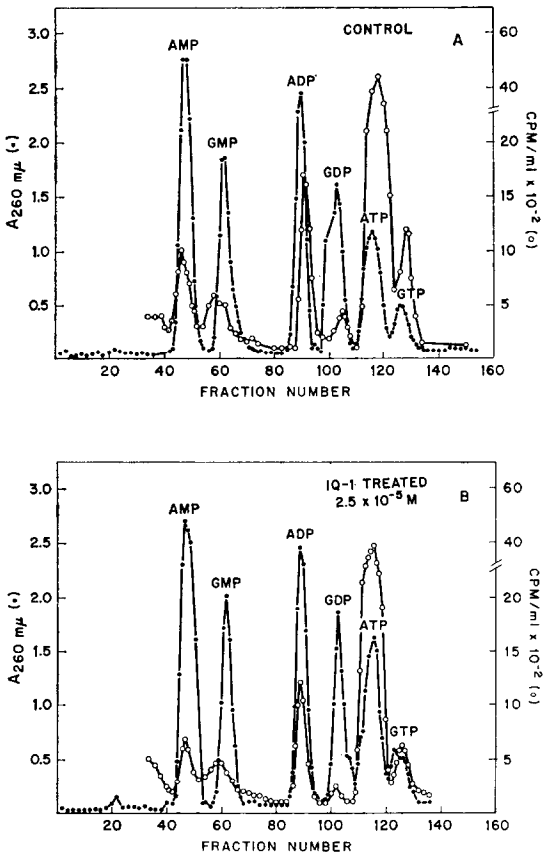


FIG. 2. Effect of IQ-1 on the incorporation of ^{3}H-adenosine into acid-soluble purine nucleotides of *E. coli* B. The experimental details were as described in Fig. 1, except that ^{3}H-adenosine (8 μCi/μmole) was added to each flask at a final concentration of 25 μM. A, untreated control; B, IQ-1-treated.

nucleoside monophosphate kinase. A partially purified preparation of nucleoside monophosphate kinase, capable of catalyzing a phosphotransferase reaction using either UMP, CMP, AMP, UDP, CDP or ADP as substrates was employed to corroborate the evidence that IQ-1 interfered with the activity of pyrimidine nucleoside monophosphate kinase. The results in Table 2 show the effects of the heterocyclic carboxaldehyde thiosemicarbazone on nucleotide kinase activity using CMP as substrate. IQ-1 (5×10^{-5} M) caused a 47 per cent depression of the conversion of ^{14}C-CMP to CDP and CTP, indicating strong blockade of this enzymic reaction. That the effects of the drug are exerted upon pyrimidine nucleoside monophosphate

TABLE 2. EFFECT OF IQ-1 ON NUCLEOTIDE KINASE ACTIVITY UTILIZING ^{14}C-CMP AS SUBSTRATE*

Treatment	Incubation time (min)	Radioactivity (counts/min)		
		CMP	CDP	CTP
None	7	2710	140	370
None	15	1940	270	1050
IQ-1 (50 μM)	15	2770	120	580
Heat-inactivated enzyme	15	3660	110	60

* Nucleotide kinase assays utilized 2 μmoles ^{14}C-CMP (50 μCi/mmole) and incubations were carried out at 37° for the indicated times. Enzyme, 25 μl, was used in all assays. The reaction was terminated by the addition of an equal volume of cold 8% PCA, followed by filtration, neutralization with KOH and chromatographic separation. The final neutralized supernatant volume was 1·95 ml for each assay. Samples, 40 μl, were applied to PEI-cellulose plates to separate nucleotides and radioactivity in various fractions was determined.

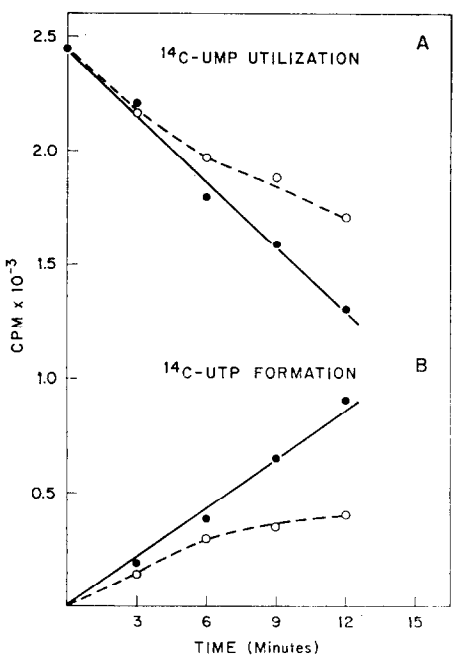


FIG. 3. Effect of IQ-1 on nucleotide kinase activity using ^{14}C-UMP as substrate. Nucleotide kinase assays utilized 6 μmoles ^{14}C-UMP (2 mCi/mmole) as substrate. Incubations were carried out at 37° for the indicated times. The reaction was terminated with an equal volume of cold 8% PCA, followed by filtration, neutralization and chromatographic separation using PEI-cellulose thin-layer sheets. The incubation mixture contained a volume of 4 ml and 0·5-ml samples were removed and assayed at each time point. Two hundred μl (0·7 mg protein) of enzyme preparation is contained in each 4 ml of assay mix. Open circles (○——○), IQ-1-treated (2·5 × 10^{-5} M) samples; closed circles (●——●), control values. The upper half of the figure (A) depicts ^{14}C-UMP utilization and the lower half (B) shows the rate of formation of ^{14}C-UTP.

kinase activity is shown by the potent inhibition by IQ-1 of the enzymatic conversion of ^{14}C-UMP to UTP (Fig. 3).

The effects of IQ-1 upon nucleoside monophosphate kinase using ^{14}C-AMP as substrate were also measured. The results shown in Fig. 4, A and B, indicate that 5×10^{-5} M IQ-1 did not significantly interfere with either the utilization of AMP by

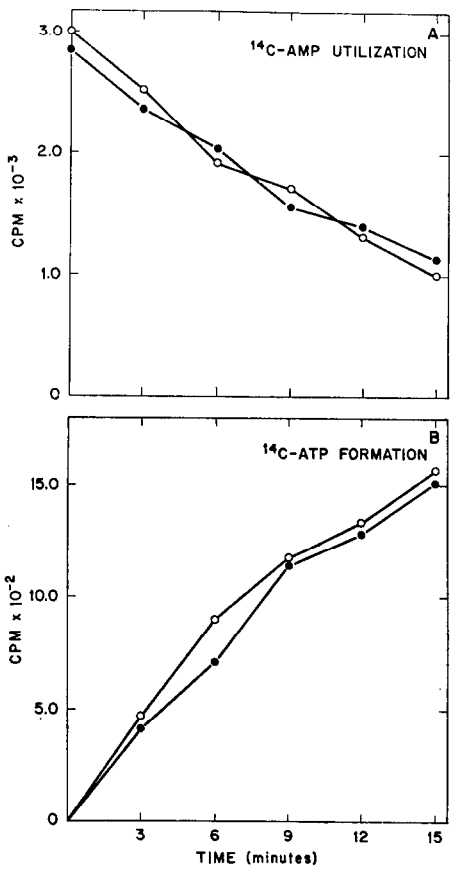


FIG. 4. Effect of IQ-1 on nucleotide kinase activity using ^{14}C-AMP as substrate. The experimental details were as described in Fig. 3, except that 3·2 μmoles ^{14}C-AMP (2 mCi/mmole) were employed as substrate. Closed circles (●——●), IQ-1-treated (5×10^{-5} M) samples; open circles (○——○), control values. The upper half of the figure (A) depicts ^{14}C-AMP utilization and the lower half (B) shows the rate of formation of ^{14}C-ATP.

the enzyme system or the appearance of radioactivity in ATP, the product of the reaction. Thus, a site of action of IQ-1 in *E. coli* appears to be at the enzyme(s) pyrimidine nucleoside monophosphate kinase.

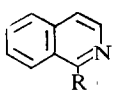
To gain evidence that interference with the activity of pyrimidine nucleoside monophosphate kinase was involved in the bacteriostatic potency of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones in *E. coli*, the effects of several derivatives of this class with varying growth-inhibitory potencies were measured on the enzyme (Table 3). The weaker growth-inhibitory agents, 1-formylisoquinoline guanylhydrazone

(—CH=NNHC(=NH)NH_2) and 1-formylisoquinoline semicarbazone (—CH=NNHCONH_2), caused less depression of enzymic activity than did IQ-1, whereas derivatives containing a morpholino

(—CH=NNHCSN⟨ ⟩O)

or a methylhydrazino (—CH=NNHCSNHNHCH_3) group, which were more potent antimicrobial agents than IQ-1, caused greater interference with pyrimidine nucleoside monophosphate kinase activity. However, the absence of a perfect correlation between the abilities of the compounds to interfere with growth and kinase activity, as well as previous results demonstrating weak inhibition of ^{14}C-adenine into RNA,[7] indicates the involvement of other factors in the growth-inhibitory action of IQ-1 in *E. coli*.

TABLE 3. EFFECT OF MODIFICATION OF THE THIOSEMICARBAZONE SIDE CHAIN ON THE GROWTH AND INHIBITION OF NUCLEOSIDE MONOPHOSPHATE KINASE ACTIVITY OF *E. coli*

 R	% Inhibition	
	Growth*	Nucleoside monophosphate kinase activity†
—CH=NNHCSNH_2‡	68	51
—CH=NNHC(=NH)NH_2	27	9
—CH=NNHCONH_2	12	30
—CH=NNHCSN⟨ ⟩O	100	>95
—CH=NNHCSNHNHCH_3	75	>95

* 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.

† Nucleoside monophosphate kinase activity was measured using ^{14}C-UMP as the substrate, as described in Fig. 3.

‡ 1-Formylisoquinoline thiosemicarbazone (IQ-1).

The inability of IQ-1 to interfere with the conversion of adenosine to ATP suggested that the slight depression of the incorporation of the purine ribonucleoside into RNA was the result of inhibition of RNA polymerase. The effectiveness of IQ-1 as an inhibitor of RNA polymerase appeared to depend upon the preparation employed; however, no specific type of preparation appeared to show consistent sensitivity or resistance to IQ-1 regardless of whether the method of Burgess[10] or the modified procedure of Chamberlain and Berg[11] was employed. Thus, thirteen different enzyme preparations were tested; six of these showed sensitivity of a significant degree (greater than 50 per cent inhibition by 5×10^{-5} M IQ-1). An example of the dose–response of a sensitive preparation is shown in Table 4. The concentration of IQ-1 causing 50 per cent inhibition of bacterial growth reduced polymerase activity to 31 per cent of control activity. Preparations inhibited by IQ-1 were always sensitive to this agent when retested, even after several weeks of storage; in a like manner, insensitive RNA polymerase enzymes were always resistant. Differences in response to α-(*N*)-heterocyclic carbox-

TABLE 4. EFFECT OF VARYING CONCENTRATIONS OF IQ-1 ON RNA POLYMERASE

Concentration (M)	% Activity
1×10^{-6}	62
1×10^{-5}	50
$2{\cdot}5 \times 10^{-5}$	31
1×10^{-4}	19

* Fifty-four μg RNA polymerase (Chamberlain–Berg enzyme) was added to the assay mixture described in Materials and Methods; the final volume of the reaction was 250 μl. Incubations were carried out at 37° with shaking for 10 min, after which an equal volume of cold 10% TCA was added to terminate the reaction, and the rate of incorporation of ^{14}C-ATP into acid-precipitable material was determined.

aldehyde thiosemicarbazones were not due to differences in relative enzyme activity, the quantity or source of the DNA template, the salt concentration, the type of sulfhydryl reagent employed, enzyme concentration or pH. It appeared that crude supernatant extracts (cells ground with glass beads and centrifuged at 30,000 *g* for 10 min) from

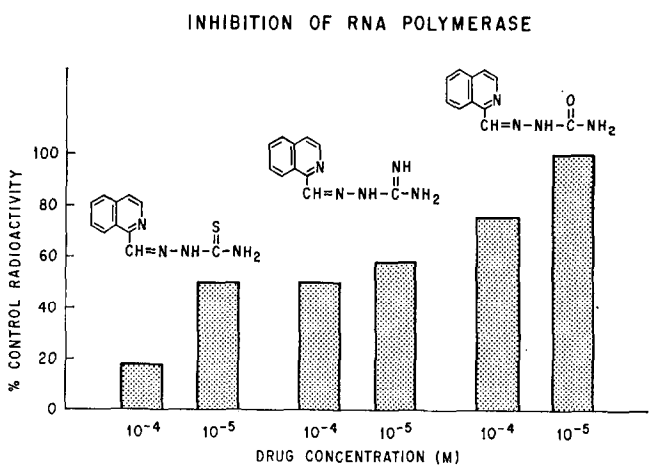


FIG. 5. Effects of IQ-1, 1-formylisoquinoline guanylhydrazone and 1-formylisoquinoline semicarbazone on RNA polymerase activity. Assays were performed as described in Materials and Methods.

most preparations were inhibited by IQ-1. After centrifugation at 100,000 *g* for 2 hr, preparations were either sensitive or acted in an unpredictable fashion to inhibition by this agent. Attempts to reconstitute fractions to restore sensitivity to drug-resistant preparations were unsuccessful.

The effects of two analogs of IQ-1 with lesser ability to inhibit growth were tested for their potencies as inhibitors of RNA polymerase using an enzyme preparation sensitive to IQ-1. The structures of these agents and their effects on RNA polymerase are illustrated in Fig. 5. 1-Formylisoquinoline guanylhydrazone was considerably less potent than IQ-1, and the semicarbazone was only slightly effective at the higher concentration employed. These results correlated well with the relative potencies of these agents as inhibitors of the growth of *E. coli* B, as is shown in Table 3.

DISCUSSION

Previous findings[7] led to the hypothesis that the bacteriostatic activity of IQ-1 in *E. coli* was a consequence of both a general blockade of RNA synthesis and a second more pronounced site of action on the pathways involved in pyrimidine nucleotide metabolism. Studies with DNA-directed RNA polymerase of *E. coli* have demonstrated that under certain conditions the enzyme is sensitive to the inhibitory activity of IQ-1; inhibition of RNA polymerase would appear sufficient to account for the monitored general interference with the formation of RNA. The exact mechanism by which IQ-1 inhibits the activity of this enzyme is unknown and must await future purification, characterization and stabilization of the properties of the enzyme; however, simple drug interaction with the DNA template, which can be demonstrated by spectral changes,* does not seem to be involved in the mechanism of inhibition of this enzymic reaction.

The interference by IQ-1 of the conversion of UMP to di- and triphosphates in intact cells and of UMP and CMP to the triphosphate level in a partially purified preparation of nucleotide kinase accounts for the observed effects of this agent on the incorporation of radioactive precursors of pyrimidine nucleotide metabolism into RNA.[7]

The metabolic pathways for the formation of the nucleoside triphosphates necessary for RNA biosynthesis are relatively well established, requiring as an initial step the conversion of nucleoside monophosphates to the triphosphate level via a phosphotransferase reaction. Since no known alternate routes are available for the synthesis of higher pyrimidine nucleotide forms, the essentiality of these enzymes places them in a powerful position with respect to their ability to control nucleic acid synthesis. The inhibition of pyrimidine nucleoside monophosphate kinase activity by α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, therefore, results in a lesion of major consequence to the synthesis of RNA.

Structure–activity relationships, although limited, have suggested the importance of pyrimidine nucleoside monophosphate kinase and RNA polymerase in the bacteriostatic action of the heterocyclic carboxaldehyde thiosemicarbazones. The most active inhibitors of nucleotide kinase were the derivatives of IQ-1 containing a morpholino or a methylhydrazino group. Evidence that similar blockade of nucleotide kinase activity of Sarcoma 180 by IQ-1 occurs and is involved in the inhibition of RNA synthesis by this agent in mammalian cells was suggested by the finding that the IQ-1 derivative containing the morpholino group caused a greater degree of inhibition of RNA relative to DNA than did the parent molecule.[14]

Inhibition of pyrimidine nucleoside monophosphate kinase by IQ-1 represents the first example of an agent inhibitory to this enzyme and, if a similar lesion occurs in

* A. C. Sartorelli, unpublished observation.

other cell lines, this agent may represent a useful biochemical tool to manipulate in a reversible manner the biosynthesis of RNA in microbial cells.

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