Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.
Deshmukh, A. D., and Nimni, M. E. (1969), *Biochem. Biophys. Res. Commun. 35*, 845.
Folk, J. E., and Gladner, J. A. (1960), *J. Biol. Chem. 35, 60.*
Gross, J. (1969), *Aging Connective Skeletal Tissue, Thule Int. Symp., 1968*, 33.
Hayaishi, O. (1963), *Enzymes 8*, 353.
Hayaishi, O. (1969), *Annu. Rev. Biochem. 38*, 21.
Kimball, D. A., Coulson, W. F., and Carnes, W. H. (1964), *Exp. Mol. Pathol. 3*, 10.
Martin, G. R., Gross, J., Piez, K. A., and Lewis, M. S. (1961), *Biochim. Biophys. Acta 53*, 599.
Martin, G. R., Pinnell, S. R., Siegel, R. C., and Goldstein, E. R. (1970), *in* Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 1, Balazs, E. A., Ed., New York, N. Y., Academic Press, p 403.
Miller, E. J., Martin, G. R., Mecca, C. E., and Piez, K. A. (1965), *J. Biol. Chem. 240*, 3623.
O'Dell, B. L., Hardwick, B. C., Reynolds, G., and Savage, J. E. (1961), *Proc. Soc. Exp. Biol. Med. 108*, 402.
Piez, K. A. (1968), *Annu. Rev. Biochem. 37*, 547.
Pinnell, S. R., and Martin, G. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 708.
Schiffmann, E. S., and Martin, G. R. (1970), *Arch. Biochem. Biophys. 138*, 226.
Shields, G. S., Coulson, W. F., Kimball, D. A., Carnes, W. H., Cartwright, G. E., and Wintrobe, M. M. (1962), *Amer. J. Pathol. 41*, 603.
Siegel, R. C., and Martin, G. R. (1970a), *J. Biol. Chem. 245*, 1953.
Siegel, R. C., and Martin, G. R. (1970b), *Calcif. Tissue Res. 4*, 42.
Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Symp. Biol. 1*, 91.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1960), *J. Biol. Chem. 235*, 64.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

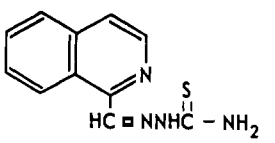
Inhibition of Ribonucleoside Diphosphate Reductase by 1-Formylisoquinoline Thiosemicarbazone and Related Compounds*

E. Colleen Moore, Morris S. Zedeck,† Krishna C. Agrawal, and Alan C. Sartorelli‡

ABSTRACT: Interference with the biosynthesis of DNA by 1-formylisoquinoline thiosemicarbazone (IQ-1) was shown to be due to inhibition of ribonucleoside diphosphate reductase. The molecular mechanism of the inhibition by IQ-1 was investigated with an enzyme from a rat tumor, purified approximately 20-fold over the 100,000*g* supernatant fraction. The concentration of the nucleoside diphosphate substrate, the allosteric activator ATP, or magnesium ion did not influence the inhibition by IQ-1. Dithioerythritol or dithiothreitol, model dithiols used in place of the natural substrate thioredoxin (dithiol form), were partially competitive with the inhibitor. Although IQ-1 has a great affinity for ferrous ion, the inhibition of the enzyme was not reversed, and in certain conditions was enhanced, by increasing the concentration of this cation. The findings are compatible with a model in which either IQ-1 binds to an iron-charged enzyme or the iron chelate of IQ-1 interacts with the enzyme at the site occupied by the dithiol substrate.

A number of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones that form coordination compounds with iron, cobalt, nickel, copper, zinc, and manganese have been shown to be potent inhibitors of the growth of a variety of transplanted rodent neoplasms (Sartorelli and Creasey, 1969). Isoquinoline derivatives are among the most effective tumor inhibitors in this class, one of the most potent agents being 1-formylisoquinoline thiosemicarbazone (IQ-1)[1] (structure I) (Sartorelli *et al.*, 1968). The precise biochemical site

I



* From the Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025, and Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received February 18, 1970.* This work was supported in part by Grants CA-02817 and CA-04464 from the National Cancer Institute, U. S. Public Health Service, and Grant T-23 from the American Cancer Society.

† Present address: Division of Pharmacology, Sloan-Kettering Institute for Cancer Research, New York, N. Y. 10021.

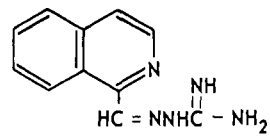
‡ Inquiries concerning this manuscript should be made to Dr. A. C. Sartorelli, Department of Pharmacology, Yale University School of Medicine.

[1] The following abbreviations are used: [^{32}P]CDP, [α-^{32}P]cytidine diphosphate; DTE, dithioerythritol; DTT, dithiothreitol; IQ-1, 1-formylisoquinoline thiosemicarbazone.

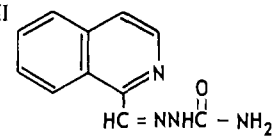
of action of IQ-1 is unknown; however, earlier reports from this laboratory (Sartorelli, 1967a,b) have shown that the biosynthesis of DNA is extremely sensitive to the inhibitory action of IQ-1. Blockade of the formation of RNA and protein also occurs, but these processes are considerably less susceptible to this inhibitor.

This report presents evidence that the reduction of ribonucleotides to deoxyribonucleotides is inhibited by IQ-1; interference with this conversion was due to inhibition of ribonucleoside diphosphate reductase. The molecular mechanism of action of IQ-1 was investigated with a partially purified reductase preparation from a rat tumor; the data obtained are compatible with a model in which the metal binding agent IQ-1 interacts with the iron-containing form of the enzyme or a preformed iron chelate of IQ-1 binds to the enzyme. The activity of the guanylhydrazone and semicarbazone analogs of IQ-1 (structures II and III) was compared to that of the thiosemicarbazone in both the whole cell and the enzyme systems. A preliminary report of these findings has appeared (Sartorelli *et al.*, 1968).

II



III



Materials and Methods

Chemicals. CDP labeled in the α-phosphate with ^{32}P was prepared in our laboratory from [α-^{32}P]CTP purchased from Schwarz BioResearch. The labeled CTP was incubated with partially purified nucleoside diphosphate kinase (EC 2.7.4.6) (Ratliff *et al.*, 1964) and an excess of adenosine diphosphate. The product was isolated by the procedures of Hurlbert and Furlong (1967). Other labeled and unlabeled nucleotides were commercial products. 1-Formylisoquinoline thiosemicarbazone was donated by Mr. Frederic A. French, Cancer Chemotherapy Research Department, Mount Zion Hospital and Medical Center, San Francisco, Calif., and Dr. Harry B. Wood, Jr., Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. 1-Formylisoquinoline semicarbazone and 1-formylisoquinoline guanylhydrazone were synthesized as previously described (Agrawal and Sartorelli, 1969).

Studies with Intact Cells. Six-day-old Sarcoma 180 ascites cells (approximately 1.5×10^8 cells) were incubated for 30 min at 37° in a total volume of 12 ml of Fischer's medium (Fischer and Sartorelli, 1964) minus horse serum with inhibitors and either [^3H]thymidine (160 μg, 2.1×10^4 cpm/μg) or [5-^3H]-cytidine (160 μg, 1.2×10^4 cpm/μg). Inhibitors were solubilized in dimethyl sulfoxide; the final concentration of solvent, which did not inhibit the conversion of radioactive precursors into deoxynucleotide forms, did not exceed 4%. In experiments with [^3H]thymidine as precursor, the reaction was terminated by the addition of $HClO_4$ to give a final concentration of 0.4 M. The precipitate was washed twice with 0.2 M $HClO_4$, extracted with 0.4 M $HClO_4$ at 90° for 15 min, and aliquots were analyzed both for radioactivity with a Packard Tri-Carb liquid scintillation spectrometer and for deoxyribose using deoxyadenosine as the standard (Schneider, 1955). In experiments with [5-^3H]cytidine as precursor, the cells were collected by centrifugation, the cold $HClO_4$-soluble fraction was collected quantitatively and neutralized with KOH, and the $KClO_4$ was removed by centrifugation. The resulting supernatant was concentrated and incubated for 1 hr at 37° with 0.6 M Tris-HCl (pH 8.5)–0.06 M $MgSO_4$ and lyophilized crude *Crotalus adamenteus* venom extract (1 mg/ml). The reaction was terminated by the addition of cold acetone–glacial acetic acid (9:1, v/v) and the mixture was centrifuged. The supernatant was chromatographed (descending) on Whatman No. 1 paper using absolute ethanol–5 M ammonium acetate (pH 9.5)–saturated sodium tetraborate–0.5 M EDTA (110:10:40:0.25, v/v) as described by Reichard (1958). The areas corresponding to entire cellular ribonucleotide and deoxyribonucleotide pools were removed and radioactivity was determined by direct liquid scintillation counting of paper strips.

Conversion of [^3H]Thymidine into Acid-Soluble Deoxynucleotides. Mice bearing 6-day growths of Sarcoma 180 ascites cells were given a single intraperitoneal injection of IQ-1. Twelve hours later 200 μg of [^3H]thymidine (6.7×10^3 cpm/μg) was administered by injection and 1 hr was allowed for metabolism. Cold 4% $HClO_4$-soluble extracts of these cells were neutralized to pH 7 with potassium hydroxide, potassium perchlorate was removed by centrifugation, and a 5-ml portion of the supernatant was subjected to chromatography on 1.4×9 cm columns of Ecteola-cellulose (exchange capacity approximately 0.5 mequiv/g). Thymidine was eluted with water, thymidine monophosphate with 0.01 N HCl, and thymidine di- and triphosphates with 0.5 N HCl. The concentration of thymidine or thymine nucleotide in each fraction was determined by measurement of the radioactivity therein. Sodium nucleates were isolated by the method of Tyner *et al.* (1953) and hydrolyzed with 70% $HClO_4$ for 1.5 hr (Marshak and Vogel, 1951). Extracts were desalted on charcoal columns, and DNA-thymine was purified and analyzed as described by Danneberg *et al.* (1958).

Enzymes and Assays. Sarcoma 180 ascites cells, harvested at 7 days and washed once by centrifugation with 0.9% NaCl, were suspended in 1.5 volumes of 0.02 M Tris-HCl buffer (pH 7) and disrupted by four 15-sec exposures to ultrasonic vibration with a Bronson power probe sonifier at a power setting of 5. The homogenate was centrifuged 50 min at 100,000g and the supernatant was used as the enzyme source for ribonucleotide reductase assays. The assay mixture was that described for crude extracts (Moore, 1967a), except that the magnesium acetate concentration was doubled and ferrous ammonium sulfate 2.3×10^{-5} M was substituted for ferric chloride.

The ribonucleoside diphosphate reductase of the Novikoff rat tumor was purified and assayed as previously described (Moore, 1967a), except for variations in the incubation mixture. Several batches of enzyme were used. The purification varied from 12- to 50-fold over the 100,000g supernatant fraction, and specific activities ranged from 12 to 72 nmoles of CDP reduced per hr per mg of protein. The preparation

TABLE I: Effect of Inhibitors on the Incorporation of [^3H]-Thymidine into DNA of Sarcoma 180 Cells *in Vitro*.[a]

Inhibitor Concn (M)	% Control Specific Activity		
	IQ-1	1-Formylisoquinoline Guanylhydrazone	1-Formylisoquinoline Semicarbazone
1×10^{-7}	72 (68–77)[b]	*c*	*c*
2×10^{-7}	56 (55–58)	*c*	*c*
4×10^{-7}	34 (30–53)	95 (71–103)	96 (87–104)
4×10^{-6}	12 (11–13)	89 (72–96)	90 (76–100)
4×10^{-5}	*c*	80 (67–89)	90 (84–102)
4×10^{-4}	*c*	24 (21–28)	72 (72–73)

[a] The specific activity of DNA of control cells was 2.5×10^3 cpm/μmole of deoxyribose. [b] Each value represents the average of results from two to four flasks with the range given in brackets. [c] Not determined at indicated concentration.

TABLE II: Effect of IQ-1[a] on the Conversion of [^3H]Thymidine into Acid-Soluble Deoxynucleotides.

IQ-1[b] (mg/kg)	Total Acid Soluble (cpm $\times 10^{-3}$)	% of Total Acid-Soluble Radioactivity			Sp Act. of DNA (cpm/μmole $\times 10^{-2}$)
		dTR	dTMP	dTDP + dTTP	
None	104.8	64.6	16.2	19.2	112.6
3.0	111.4	66.2	10.8	23.0	60.7
25	139.3	63.0	6.6	30.4	0.6

[a] Abbreviations: IQ-1, 1-formylisoquinoline thiosemicarbazone; dTR, deoxythymidine; dTMP, deoxythymidine monophosphate; dTDP, deoxythymidine diphosphate; dTTP, deoxythymidine triphosphate. [b] Mice bearing 6-day growths of Sarcoma 180 ascites cells were injected i.p. with the indicated concentration of IQ-1. Twelve hours later, [^3H]thymidine (200 μg/animal; 6.7×10^3 cpm/μg) was administered and 1 hr was allowed for metabolism. Each value represents the average of results obtained with four mice.

usually contains significant amounts of nucleoside diphosphate kinase and thioredoxin. Other contaminating enzymes are not known, but when the recovery of labeled CDP has been checked after incubation with the enzyme, essentially all of the label is accounted for as cytidine and deoxycytidine nucleotides. The iron content of the enzyme preparations is sufficient to contribute 0.5–2.0 μM iron to the incubation mixture; part of this is probably present as hemoglobin.

$Fe(NH_4)_2(SO_4)_2$ was substituted for $FeCl_3$ in most experiments at a concentration of 4×10^{-6} M, unless otherwise stated. Dithiothreitol (DTT) was used instead of dithioerythritol (DTE) in a few experiments because the batch available contained less contaminating iron; identical concentration curves were obtained with the two dithiols. In the experiments in which an NADPH-linked system was used, DTE was omitted and NADPH (4×10^{-4} M), NAD^+ (2×10^{-4} M), glucose 6-phosphate (3×10^{-4} M), glucose 6-phosphate dehydrogenase (0.1 unit/ml), and reduced glutathione (1.6×10^{-3} M) were included, together with thioredoxin and thioredoxin reductase from *Escherichia coli* (Laurent *et al.*, 1964; Moore *et al.*, 1964) or the equivalent system from the rat tumor (Moore, 1967b). Thioredoxin was a gift of Professor Peter Reichard; the other proteins were partially purified preparations from our laboratory. Bacterial ribonucleotide reductase was prepared as described (Reichard, 1962). [^{32}P]CDP was used as substrate in most experiments; it was diluted to specific activities of $1–1.5 \times 10^6$ cpm/μmole. ^{14}C-Labeled UDP and GDP were used in one experiment each; with GDP as substrate the appropriate allosteric activator deoxythymidine triphosphate was included (Moore, 1967a). The inhibitors were dissolved in dimethyl sulfoxide; the maximum concentration of solvent in the incubation mixture was 1% and was not inhibitory.

Sephadex Treatment. After incubation of enzyme solution for 15 min with inhibitor, DTE, and Fe^{2+}, 0.9 ml of the mixture was added to a column of Sephadex G-25 (1×20 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7) and eluted with the same buffer. The effluent was monitored with an ISCO double-beam ultraviolet recorder operated at 280 mμ; effluent (1.5 ml) having the highest A_{280} was collected. The DTE from the enzyme solution was clearly separated from the protein as detected by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). After all the DTE was eluted, the second protein sample was added and eluted in an identical manner. The concentration of protein in the eluates was measured as A_{280} with a Zeiss spectrophotometer.

Calculation of Kinetic Constants. The apparent Michaelis constants and maximum velocities were calculated by computer according to Cleland (1963a), except in the case of the constants for magnesium and DTT which were determined graphically. The 50% inhibitory concentration of drugs was determined graphically (Moore, 1969).

Results

The primary biochemical lesion created by IQ-1 *in vivo* was shown to be inhibition of the synthesis of DNA (Sartorelli, 1967a,b); such blockade was also expressed in intact cells *in vitro* (Table I). Thus, the incorporation of [^3H]thymidine into DNA was inhibited approximately 50% by a concentration of IQ-1 of 2×10^{-7} M. To achieve a comparable degree of inhibition $>4 \times 10^{-5}$ M 1-formylisoquinoline guanylhydrazone or $>4 \times 10^{-4}$ M 1-formylisoquinoline semicarbazone were required.

To assist in the localization of the metabolic lesion produced by IQ-1 on the DNA biosynthetic pathways, the conversion of thymidine to the nucleotide level was measured in Sarcoma 180 cells at levels of IQ-1 that inhibit the synthesis of DNA (Table II). No decrease in the conversion of thymidine into nucleotide forms was produced in cells pretreated with IQ-1. Rather, the quantity of radioactivity found in thymidine di- and triphosphates appeared to be elevated in drug-treated cells. These results suggested that the inhibition of DNA synthesis was due either to interference

TABLE III: Effect of Inhibitors on the Conversion of [5-^3H]-Cytidine into Acid-Soluble Nucleotides of Sarcoma 180 Cells *in Vitro*.[a]

Inhibitor	Concn (M)	% Control Incorporation	
		Ribonucleotides	Deoxyribonucleotides
IQ-1	4×10^{-7}	90,116[b]	19,36
	4×10^{-6}	91,99	18,24
	4×10^{-5}	77,96	10,14
1-Formylisoquinoline guanylhydrazone	4×10^{-5}	88,89	57,75
	2×10^{-4}	93,100	20,24
1-Formylisoquinoline semicarbazone	2×10^{-4}	90,96	113,138

[a] The ribonucleotide and deoxyribonucleotide pools of control cells contained 30,500–36,500 and 1050–1150 cpm, respectively, after incubation for 30 min with [5-^3H]cytidine. [b] Each number represents the per cent of control incorporation of a separate flask.

TABLE IV: Effect of IQ-1 on Reduction of CDP by Sarcoma 180 Extract.[a]

IQ-1 (10^{-7} M)	mμmoles/30 min	
	Expt 1	Expt 2
None	0.56	0.74
0.32	0.45	0.65
0.54	0.31	0.52
1.07	0.20	0.41
1.6	0.16	0.35
2.1	0.14	0.30
3.2	0.10	0.21
4.8	0.08	0.17

[a] Activity is expressed as millimicromoles of CDP reduced in 30 min. Each tube contained 50 μl of Sarcoma 180 extract (0.8 mg of protein) in a total volume of 0.12 ml. In expt 1, the mixture contained 1.2×10^{-3} M DTT; in expt 2, the concentration was 6.3×10^{-3} M.

with the polymerization of deoxynucleotides, or to inhibition of the production of one or more deoxyribonucleoside triphosphates. That IQ-1 interfered with the synthesis of deoxyribonucleotides is shown in Table III. The incorporation of [5-^3H]cytidine into the cellular pool of ribonucleotides was relatively unaffected by IQ-1; however, the incorporation of label into the deoxyribonucleotide pool was markedly inhibited. In this regard, 1-formylisoquinoline guanylhydrazone was considerably less effective, and 1-formylisoquinoline semicarbazone was not inhibitory at a concentration of 2×10^{-4} M.

The findings suggested that the site of blockage of deoxyribonucleotide synthesis was at the level of the enzyme ribonucleoside diphosphate reductase. This hypothesis was supported by the *in vitro* inhibition of reduction of CDP by a high-speed supernatant prepared from Sarcoma 180 cells (Table IV). IQ-1 at an estimated concentration of 7×10^{-8} M inhibited the reduction of CDP by 50% when the concentration of DTT was 1.2×10^{-3} M; at 6.3×10^{-3} M DTT, approximately 1.4×10^{-7} M IQ-1 was required for the same degree of inhibition.

In order to study the inhibition in greater detail, a partially purified ribonucleotide reductase prepared from the Novikoff rat tumor was used. Under standard conditions of assay the reduction of CDP by this enzyme was inhibited 50% by approximately 5×10^{-8} M IQ-1 (Table V), although some variation between experiments was observed. Similar results have been reported (Brockman *et al.*, 1970) with another enzyme preparation. In our experience, no other inhibitor of this enzyme has been active at so low a concentration. Fifty per cent inhibition of enzyme activity required 4×10^{-5} M 1-formylisoquinoline guanylhydrazone, while a comparable degree of inhibition by 1-formylisoquinoline semicarbazone was estimated graphically to require 4×10^{-4} M. Inhibition was not unique to the substrate CDP; thus, the reduction of UDP and GDP was inhibited to approximately the same extent by IQ-1.

No competition was detected between IQ-1 and the ribonucleotide substrate (CDP), allosteric activator (ATP), or Mg^{2+}; apparent Michaelis constants and maximum velocities in the presence and absence of IQ-1 are shown in Table VI.

A relationship was observed between inhibition of ribonucleoside diphosphate reductase by IQ-1 and the concentration of the reducing substrate DTE or DTT. The dithiols were used as a convenient substitute for the natural thioredoxin–thioredoxin reductase–NADPH system. Variation in the concentration of DTE has been shown to affect the activity of the enzyme, the requirement of the enzyme for ferrous ion, and the degree of inhibition by hydroxyurea (Moore, 1969). No difference has been observed between the effects of DTE and DTT on the enzyme system.

TABLE V: Effect of IQ-1, 1-Formylisoquinoline Guanylhydrazone, and 1-Formylisoquinoline Semicarbazone on the Activity of Ribonucleoside Diphosphate Reductase.[a]

Inhibitor	ID_{50} (M)
1-Formylisoquinoline thiosemicarbazone (IQ-1)	5×10^{-8}
1-Formylisoquinoline guanylhydrazone	4×10^{-5}
1-Formylisoquinoline semicarbazone	4×10^{-4}[b]

[a] Each inhibitor was tested at several concentrations up to 10^{-4} M with a single preparation of partially purified ribonucleoside diphosphate reductase under standard conditions. The 50% inhibitory level (ID_{50}) was determined graphically. [b] Estimated.

TABLE VI: Apparent Kinetic Parameters in the Presence and Absence of IQ-1.[a]

Reactant	IQ-1 (10^{-8} M)	K_m (10^{-4} M)	V_{max} (mμmoles/30 min)
ATP		3.8 ± 0.8	2.79 ± 0.28
	1.8	3.7 ± 0.8	2.21 ± 0.23
	3.6	3.6 ± 0.7	1.72 ± 0.17
CDP		0.18 ± 0.04	0.54 ± 0.04
	2.4	0.21 ± 0.03	0.39 ± 0.02
	5.5	0.15 ± 0.01	0.18 ± 0.004
Mg^{2+}		13	1.25
	5.6	13	0.48

[a] It should be noted that these are only apparent values, as CDP is in equilibrium with CTP, ATP is an activator rather than a substrate, and Mg^{2+} may be present in the enzyme preparation.

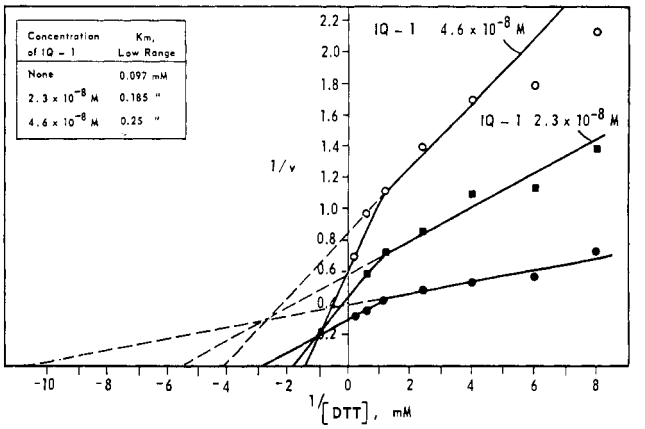


FIGURE 2: Reciprocal plot of DTT concentration *vs.* velocity, as affected by IQ-1. The enzyme was preincubated for 10 min in a volume of 90 μl with dithiothreitol and iron. Then 30 μl of mix containing ATP, buffer, Mg^{2+}, [^{32}P]CDP, and inhibitor was added and the samples were incubated an additional 30 min. The concentrations are given for the final incubation mixture. The Fe^{2+} concentration was 6 μM. Velocity units are millimicromoles reduced in 30 min.

Inhibition of the reduction of CDP by IQ-1 was more pronounced at a low dithiol concentration than at a higher one (Figure 1). The degree of inhibition by IQ-1 when a very slightly purified mammalian thioredoxin system was used was approximately the same as that at 5×10^{-4} M DTE (Figure 1). Technical difficulties prevented experiments with varying concentrations of thioredoxin. To confirm the competitive relationship between IQ-1 and the dithiol, the experiment shown in Figure 2 was performed. The apparent K_m for DTT was increased in the presence of IQ-1, but the relationship was not strictly competitive since the V_{max} was decreased in the presence of inhibitor.

IQ-1 was originally synthesized as a metal chelating agent expected to have tumor-inhibitory potential (French and Blanz, 1966), and its metal coordinating properties and those of 1-formylisoquinoline guanylhydrazone and 1-formylisoquinoline semicarbazone were described (Michaud and Sartorelli, 1968). Thus, it was expected that ferrous ions would affect the pattern of inhibition produced by these agents. The addition of Fe^{2+} to the enzyme in the presence of IQ-1 did not reverse the inhibition; furthermore, the metal did not give the stimulation of enzymic activity usually observed in the absence of inhibitor (Figure 3). Partial reversal by Fe^{2+} of inhibition of reductase was obtained with the less potent inhibitor 1-formylisoquinoline guanylhydrazone (Figure 3). The K_m for ferrous ion in the experiment was $1.45 \pm 0.14 \times 10^{-6}$ M for the control, and $3.44 \pm 0.50 \times 10^{-6}$ M in the presence of 1-formylisoquinoline guanylhydrazone; the maximum velocities were 2.24 ± 0.05 and 1.17 ± 0.05, respectively.

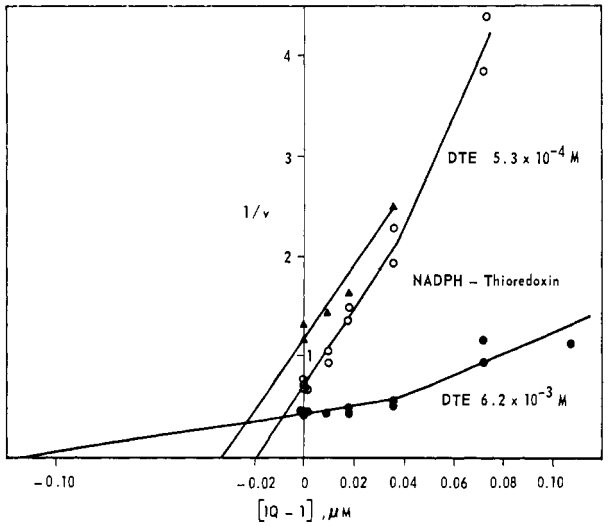


FIGURE 1: Plot of IQ-1 concentration *vs.* reciprocal velocity. Standard DTE concentration (6.2×10^{-3} M), ●—●. Low DTE (5.3×10^{-4} M), ○—○. NADPH and rat thioredoxin system in place of DTE, ▲—▲. Initial velocity (v) is given in mμmoles/30 min.

To determine whether the binding of IQ-1 to the enzyme was reversible, three types of experiments were employed. First, comparison in several experiments of inhibition by a constant level of IQ-1 with a varied enzyme level showed no consistent evidence of enzyme titration. The second approach was to determine the time course of the inhibition. The enzyme was preincubated for 10 min with ferrous ion, with or without dithiol and IQ-1. A prewarmed mixture of the remaining components of the incubation mix was then added, and samples were withdrawn at intervals for analysis. The results obtained are shown in Table VII. The activity in the first 5-min period was anomolously high; this phenomenon has been observed consistently in several experiments of this type. After the initial 5 min, the activity was linear for the controls and for the samples preincubated with IQ-1. The samples in which IQ-1 was added after the preincubation showed a gradually decreasing slope, almost equal to the control in the first 5 min, but decreasing to that of the preincubated inhibited samples for the final 20 min. The presence of DTT during preincubation may have protected the enzyme to a small extent. This experiment indicates that the inhibition

TABLE VII: Time Course of Inhibition of Ribonucleoside Diphosphate Reductase Preincubated with or without Dithiol and IQ-1.[a]

Materials Added		mμmoles			
To Preincubation Mix	After Preincubation	Incubation Time (min) 5	10	20	30
None	DTT	0.40	0.76	1.32	1.91
None	DTT + IQ-1	0.38	0.61	0.94	1.18
IQ-1	DTT	0.18	0.30	0.54	0.73
DTT	None	0.46	0.82	1.42	2.02
DTT	IQ-1	0.41	0.66	1.03	1.31
DTT + IQ-1	None	0.26	0.37	0.69	0.92

[a] Enzyme and Fe^{2+} (6 μM) were preincubated for 10 min at 37° with or without DTT and IQ-1 (3×10^{-8} M). A prewarmed mix containing reactants was added and samples were taken and analyzed for reduced product at the indicated times.

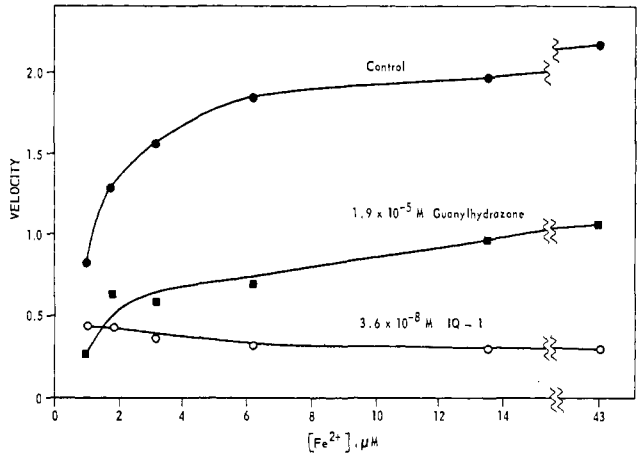


FIGURE 3: Effect of IQ-1 and 1-formylisoquinoline guanylhydrazone on the variation of activity of ribonucleoside diphosphate reductase with the concentration of ferrous ion. Standard conditions were employed except that 6×10^{-4} M DTE and various concentrations of iron were used; the indicated iron concentrations include an estimated 1 μM contributed by reagents and enzyme solution. (●—●) Control: calculated $K_m = 1.45 \pm 0.14 \times 10^{-6}$ M, $V_{max} = 2.24 \pm 0.05$. (■—■) 1.9×10^{-5} M 1-formylisoquinoline guanylhydrazone: Calculated $K_m = 3.44 \pm 0.50 \times 10^{-6}$ M, $V_{max} = 1.17 \pm 0.05$. (○—○) 3.6×10^{-8} M IQ-1. Initial velocity (v) is mμmoles/30 min.

by IQ-1 is progressive with time over the first 10 min. Last, an attempt was made to reverse the inhibition of reductase activity by IQ-1 by passing the inhibited enzyme through Sephadex G-25. Enzymatic activity was lost from the control sample by passage through Sephadex; the specific activity declined from 7.8 to 3.4 nmoles per min per mg. The activity of the sample incubated with IQ-1 was 1.4 nmoles/min per mg; on passage through Sephadex it increased slightly to 1.9. Thus the per cent inhibition was 82% before Sephadex treatment and 44% after treatment, suggesting that some inhibitor was removed.

Similarities exist between the ribonucleoside diphosphate reductase of the Novikoff tumor and *Escherichia coli* (Moore and Reichard, 1964). Nevertheless, the reduction of CDP by the ribonucleoside diphosphate reductase of *E. coli* was not inhibited by IQ-1 at concentrations up to 2.7×10^{-5} M.

Discussion

The evidence presented in this report suggests that the interference with the synthesis of DNA induced by IQ-1 is the result of inhibition of the enzyme ribonucleoside diphosphate reductase. The concentrations of IQ-1 required to inhibit both enzymatic activity and the synthesis of DNA, as well as the relative potencies of IQ-1 and the related derivatives 1-formylisoquinoline guanylhydrazone and 1-formylisoquinoline semicarbazone as inhibitors of these processes, suggest that interference with the reduction of ribonucleotides to deoxyribonucleotides is sufficient to account for blockade of the biosynthesis of DNA by these agents. The fact that the relative potencies of the compounds as inhibitors of mammalian cell proliferation (Agrawal and Sartorelli, 1969) correlate with their relative strength as inhibitors of reductase activity suggests further that blockade of the formation of DNA is responsible, at least in part, for anti-neoplastic activity.

The nature of the inhibition of ribonucleoside diphosphate reductase by IQ-1 is not completely obvious from the data. It does not appear to be influenced by either the nucleotide substrate, the allosteric activator, or magnesium (Table VI). Although we do not claim a high degree of accuracy for these kinetic constants, in view of the impurity of the enzyme preparation, it is plain that they are not significantly altered by the presence of the inhibitor. Thus it is unlikely that the inhibitor is bound at a site on the enzyme which binds either of the nucleotides. That the inhibition is at least partially reversible is suggested by the finding that passage of the IQ-1 enzyme complex through Sephadex G-25 resulted in partial restoration of enzymatic activity, which was interpreted as indicating a partial dissociation of the enzyme–inhibitor complex. However, the failure to obtain complete reversal, together with the progressive inhibition with time (Table VII), suggests the existance of an irreversible or pseudoirreversible component.

The results with ferrous ion and with the dithiol substrates show kinetic changes in the presence of IQ-1 which may offer a clue to its site of action. It is assumed for this discussion that reversibly bound ferrous ion forms a part of the active site of mammalian ribonucleoside diphosphate reductase. Stimulation of activity of the enzyme by added iron has generally been observed even in crude extracts (Moore and Hurlbert, 1962; Moore and Reichard, 1964); under some conditions ferrous ion stimulated more effectively than ferric (Moore, 1969). Although enzyme and substrates completely free of iron have not been obtained and an absolute requirement for iron has not been observed, the reduction of CDP can be inhibited at least 80% by orthophenanthroline or bipyridine and completely restored by an equimolar amount of ferrous ammonium sulfate (unpublished experiments). These observations are consistent with the assumption that reversibly bound iron plays a role analogous to that of the tightly bound iron of the *E. coli* ribonucleoside diphosphate reductase (Brown *et al.*, 1969).

Since IQ-1 chelates iron quite strongly, we expected that it would inhibit by binding the iron required by the enzyme. This did not occur; not only was the inhibition not reversed by additional iron (Figure 3), but the concentration of IQ-1 required to inhibit by 50% was only about 0.1% of the standard iron concentration. Thus binding of free iron by IQ-1 cannot play any role in the inhibition, nor can free iron prevent the inhibition by competing with the enzyme for the inhibitor.

The partially competitive relationship between IQ-1 and the dithiol substrate (Figure 2) suggests that the inhibitor may bind to the site on the enzyme normally occupied by this substrate. Similar variation of the dithiol concentration curve with IQ-1 has been observed repeatedly with both DTE and DTT, without regard to whether the enzyme was or was not preincubated with the dithiol before adding inhibitor and nucleotides. Although the K_m for dithiol varied with the conditions and to some extent with the enzyme preparation, the K_m in the presence of IQ-1 was always higher than the control value. The maximum velocity sometimes remained unchanged within experimental error, and sometimes decreased, especially at higher levels of IQ-1. Biphasic curves of the type shown have been obtained consistently with most enzyme preparations, and have also been observed by others (V. H. Bono, personal communication). We are not able to explain this phenomenon; both portions of the curve, however, indicate partial competition between IQ-1 and DTT. Failure to obtain complete reversal, either by excess dithiol or by Sephadex treatment, together with the progressive inhibition with time, may be due either to the rather tight binding of IQ-1 indicated by the low inhibition constant, or to some degree of irreversible binding of inhibitor.

It is possible that the inhibitor may interact with an enzyme–iron complex. This hypothesis is consistent with the proposed competition with dithiol, since the enzyme has a reduced requirement for iron at high dithiol levels (Moore, 1969) and *vice versa* (unpublished experiments) which is consistent with sequential binding of iron and dithiol (Cleland, 1963b). Failure to reverse the inhibition by a large excess of iron must indicate a much greater affinity of IQ-1 for the enzyme site than for free iron. Alternatively, since IQ-1 has great affinity for iron, it is possible that the active form of the inhibitor is an iron chelate of IQ-1. Such a coordination complex could bind a site normally occupied by iron and the dithiol and thereby function to abort electron transfer.

The weaker inhibition of reductase activity by 1-formylisoquinoline guanylhydrazone and 1-formylisoquinoline semicarbazone agrees with the relative potencies of these agents and that of IQ-1 as coordinators of iron (Michaud and Sartorelli, 1968). The competitive nature of the relationship between iron and 1-formylisoquinoline guanylhydrazone may be due either to coordination by the guanylhydrazone of free iron instead of, or in addition to, protein-bound iron, or to dissociation of inhibitor-bound iron from the drug–metal–enzyme complex and its replacement by free iron.

The ribonucleoside diphosphate reductase of *E. coli* contains tightly bound nonheme iron which is essential for enzyme activity (Brown *et al.*, 1969), and is inhibited by the metal binding agent hydroxyurea (Krakoff *et al.*, 1968; Moore, 1969). The failure of IQ-1 at relatively high concentrations to inhibit the reduction of CDP by this enzyme suggests that the iron is bound in such a way as to be inaccessible to the relatively bulky heteroaromatic thiosemicarbazone.

It would appear that additional understanding of the mechanism of inhibition of mammalian ribonucleoside diphosphate reductase activity by IQ-1 will require the further purification of this enzyme, as well as elucidation of the mechanism of its catalytic activity.

Acknowledgments

The authors are indebted to Odette Graham, Edward Jacquet, Niles de Grate, Cheryl Vanderford, and Andrea R. Gorske for technical assistance during this project.

References

Agrawal, K. C., and Sartorelli, A. C. (1969), *J. Med. Chem. 12*, 771.

Brockman, R. W., Sidwell, R. W., Arnett, G., and Shaddix, S. (1970), *Proc. Soc. Exp. Biol. Med. 133*, 609.

Brown, N. C., Eliasson, R., Reichard, P., and Thelander, L. (1969), *Eur. J. Biochem. 9*, 512.

Cleland, W. W. (1963a), *Nature (London) 198*, 463.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 188.

Danneberg, P. B., Montag, B. J., and Heidelberger, C. (1958), *Cancer Res. 18*, 329.

Fischer, G. A., and Sartorelli, A. C. (1964), *Methods Med. Res. 10*, 247.

French, F. A., and Blanz, E. J., Jr. (1966), *J. Med. Chem. 9*, 585.

Hurlbert, R. B., and Furlong, N. B. (1967), *Methods Enzymol. 12*, 193.

Krakoff, I. H., Brown, N. C., and Reichard, P. (1968), *Cancer Res. 28*, 1559.

Laurent, T. C., Moore, E. C., and Reichard, P. (1964), *J. Biol. Chem. 239*, 3436.

Marshak, A., and Vogel, H. J. (1951), *J. Biol. Chem. 189*, 597.

Michaud, R. L., and Sartorelli, A. C. (1968), 155th National Meeting of the American Chemical Society, San Francisco, Calif., March.

Moore, E. C. (1967a), *Methods Enzymol. 12*, 155.

Moore, E. C. (1967b), *Biochem. Biophys. Res. Comm. 29*, 264.

Moore, E. C. (1969), *Cancer Res. 29*, 291.

Moore, E. C., and Hurlbert, R. B. (1962), *Biochim. Biophys. Acta 55*, 651.

Moore, E. C., and Reichard, P. (1964), *J. Biol. Chem. 239*, 3453.

Moore, E. C., Reichard, P., and Thelander, L. (1964), *J. Biol. Chem. 239*, 3445.

Ratliff, R. L., Weaver, R. H., Lardy, H. A., and Kuby, S. A. (1964), *J. Biol. Chem. 239*, 301.

Reichard, P. (1958), *Acta Chem. Scand. 12*, 2048.

Reichard, P. (1962), *J. Biol. Chem. 237*, 3513.

Sartorelli, A. C. (1967a), *Biochem. Biophys. Res. Commun. 27*, 26.

Sartorelli, A. C. (1967b), *Pharmacologist 9*, 192.

Sartorelli, A. C., and Creasey, W. A. (1969), *Annu. Rev. Pharmacol. 9*, 51.

Sartorelli, A. C., Zedeck, M. S., Agrawal, K. C., and Moore, E. C. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 650.

Schneider, W. C. (1955), *Methods Enzymol. 3*, 680.

Tyner, E. P., Heidelberger, C., and LePage, G. A. (1953), *Cancer Res. 13*, 186.