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Purine Ribonucleotide Reductase: End-Product Inhibition and Stimulation of a Mammalian Enzyme.* (32287)

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The general area of metabolic regulation has considerable importance for studies of the oncogenic process. Specifically, control mechanisms exerted on DNA† synthesis should be of primary importance. The ribonucleotide reductases(1,2,3) catalyze the synthesis of the deoxyribonucleotides which serve as proximal precursors for DNA synthesis. Control mechanisms operating on the reductases should ultimately influence DNA synthesis. Also, chemical agents that modify the catalytic function of the reductases should interfere with the synthesis of DNA. This paper reports studies on the end-product inhibition and stimulation of purine ribonucleotide reductases, from a mouse tumor, by the natural proximal precursors for DNA. Two carcinostatic agents, β-D-arabinosylcytosine (4) and the 5′-phosphate of β-D-deoxythioguanosine(5), were also found to act as modifiers of deoxyribonucleotide formation. β-D-arabinosylcytosine (ara-c) inhibited deoxyadenylate synthesis, and deoxythioguanosine-5′-phosphate (dTGMP) was found to stimulate the formation of deoxyguanylate.

Materials and methods. Female A×C57BL/6 mice (Jackson Memorial Laboratory, Bar Harbor, Maine) were each implanted with 2×10^6 Carcinoma 755 ascites cells of a line resistant to 6-mercaptopurine. The ascites cells were harvested on day 5 and washed free of red blood cells. The ascites cells were centrifuged at 1470 × *g* for 2 minutes, diluted 1:1 with distilled water and thus subjected to osmotic shock for 2 minutes. The swollen cells were homogenized by 20 passes in a glass homogenizer with a teflon plunger. The homogenate was centrifuged at 16,000 × *g* for 90 minutes at 4°C. The crude enzyme extract was decanted and stored at —20°C. The reaction mixtures used in the enzyme tests contained AMP-8-^{14}C (Schwarz BioResearch, Inc.), 1.6×10^6 cpm/μmole, or GDP-8-^{14}C (Schwarz BioResearch, Inc.) 1.0×10^7 cpm/μmole; ATP, 0.25 μmoles; Tris-HCl, pH 7.4, 5 μmoles; $MgCl_2$,

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† Abbreviations used: DNA, deoxyribonucleic acid; ATP, adenosine-5′-triphosphate; dADP, deoxyadenosine-5′-diphosphate; ara-c, β-D-arabinosylcytosine; dGTP, deoxyguanosine-5′-triphosphate; dGDP, deoxyguanosine-5′-diphosphate; dGMP, deoxyguanosine-5′-monophosphate; dTGMP, β-D-deoxythioguanosine-5′-monophosphate; Tris-HCl, tris(hydroxymethyl) aminomethane hydrochloride buffer; AMP, adenosine-5′-monophosphate.

TABLE I. End-Product Inhibition of Adenylate Reductase by dATP.

Incubation time (min)	μμmoles dAMP formed	
	Control	5×10^{-5} M dATP added
0	0	0
15	680	310
30	1570	470
45	2010	1060

Each tube contained AMP-8-^{14}C, 1.6×10^6 cpm/μmole, 100 mμmoles; ATP, 0.25 μmoles, Tris-HCl, pH 7.4, 5.0 μmoles; $MgCl_2$, 2.5 μmoles; cell-free extract, 2.2 mg protein, in a final volume of 0.49 ml.

2.5 μmoles and crude enzyme extract equivalent to 2.0 to 3.5 mg protein in a final volume of 0.5 to 0.6 ml. Incubations were carried out at 38°C in triplicate tubes. The incubation mixtures were inactivated at the designated times by heating at 100°C for 3 minutes. Then 0.01 ml of 0.05 M KOH, 0.01 ml of 0.5 M $MgCl_2$ and 0.025 ml (0.2 mg) of crude *Crotalus adamanteus* venom were added and the incubations were continued for 60 minutes. The reactions were stopped by heating at 100°C for 3 minutes. Precipitated protein was spun down, and the supernatants were chromatographed on Whatman No. 3 MM paper, with 50 μg of the appropriate deoxyribonucleoside added as carrier in each case. The papers were developed for 24 hours in 0.64% boric acid in 85% ethanol-concentrated NH_4OH (100:1) by descending chromatography. The carrier spots were cut from the developed chromatograms and counted directly in a scintillation counter at 50% efficiency in a toluene scintillation solution. Parameters affecting this enzyme assay are described in more detail elsewhere(3). Protein was determined by the method of Lowry *et al*(6).

TABLE II. Stimulation of Deoxyadenylate Synthesis by dGTP.

	Incubation time (min)	dGTP added	μμmoles dAMP formed
Exp I	20	None	430
	"	10^{-5} M	1530
Exp II	45	None	510
	"	10^{-5} M	860

The conditions were the same as those listed under Table I. Exp I contained enzyme extract equivalent to 2.9 mg protein and Exp II contained 2.0 mg protein.

Results. Adenylate reductase. The adenylate reductase of the murine tumor was subject to end-product inhibition by dATP. The data on inhibition by dATP at 5×10^{-5} M are given in Table I. Deoxyadenylate synthesis was inhibited about 50% by this molarity of dATP. While dATP was found to inhibite deoxyadenylate synthesis, dGTP at 10^{-5} M stimulated this biosynthesis (Table II). The formation of deoxyadenylate was stimulated 360% by dGTP in a 20 minute incubation time, which is within the linear portion of the curve.

The antimetabolite, β-D-arabinosylcytosine (or metabolites), gave paradoxical inhibitory effects on the reduction of adenylate (Table III). Inhibition was reversed or partially

TABLE III. Inhibition of Adenylate Reductase by ara-c.

ara-c molarity	μμmoles dAMP formed	
	Exp I	Exp II
None	177	850
10^{-7} M	156	
5×10^{-7} M	95	
10^{-6} M	40	450
5×10^{-6} M	196	770

The conditions were the same as those listed under Table I, except that incubations were carried out for 30 min. Exp I, 2.2 mg protein; Exp II, 2.8 mg protein.

TABLE IV. Inhibition of Guanylate Reductase by dATP.

Incubation time (min)	μμmoles dGDP formed	
	Control	5×10^{-5} M dATP added
0	0	0
5	27	0
10	61	2
20	91	3

Each vessel contained GDP-8-^{14}C, 15 mμmoles; ATP, 0.25 μmoles; Tris-HCl, pH 7.4, 5.0 μmoles; $MgCl_2$, 2.5 μmoles; enzyme extract equivalent to 2.9 mg protein, in a final volume of 0.46 ml.

reversed at the highest molarity of β-D-arabinosylcytosine (ara-c) used in these studies (5×10^{-6} M).

Guanylate reductase. Guanylate reduction was almost completely inhibited by dATP at 5×10^{-5} M (Table IV). A 97 to 100% inhibition was obtained in the linear portion of the reaction. The reduction of GDP to dGDP

TABLE V. Effects of dGTP and dTGMP on Guanylate Reductase.

Incubation time (min)	μμmoles dGDP formed: Control	Modifier added 5×10^{-5} M
Exp I		dGTP added
0	0	0
5	16	1
10	53	4
20	78	9
Exp II		dTGMP added
0	0	0
5	27	44
10	71	82
20	101	112

The conditions were the same as those listed under Table IV. Enzyme extract equivalent to 3.48 mg protein was used in a final volume of 0.56 ml.

was also subject to end-product inhibition in enzyme preparations of the mammalian tumor (Table V). However, β-D-deoxythioguanosine-5′-phosphate (dTGMP), the thiol analogue of dGMP, appeared to stimulate the guanylate reductase (Table V). In fact, increases in dGDP synthesis occurred at several molarities of dTGMP (Table VI) whereas dGMP gave inhibitions at the same molarities. At the lowest molarity of modifiers employed (5×10^{-6} M), dTGMP stimulated the reaction about 50%, but dGMP gave about 50 percent inhibition.

Discussion. The inhibitory effect of dATP and the stimulatory effect of dGTP on the adenylate reductase of the Ca755 tumor are in accord with those recently reported for a purified enzyme from *Escherichia coli B.* by Larsson and Reichard(1). The reduction of GDP to dGDP was also subject to end-product inhibition in enzyme preparations of the mammalian tumor. These results, however, differ from those reported by Larsson and Reichard (1). Several interpretations of this difference are possible. (a) The observed "end-product" inhibition of the mammalian guanylate reductase may not be true end-product inhibition in the enzyme preparation. This might be dATP mediated inhibition since dGTP stimulated dATP synthesis. (b) These variances in results may reflect a true difference between the bacterial and mammalian enzymes. (c) The inhibitor site of the guanylate reductase may be sensitive to purification procedures. The inhibitor sites of some enzymes are known to be very labile(7). However, our results are similar to those reported by Moore and Hurlbert(2) for a purified guanylate reductase from the Novikoff hepatoma. The most likely explanation is that the bacterial and mammalian enzymes differ.

The inhibitory effect of β-D-arabinosylcytosine on deoxyadenylate synthesis correlates with the *in vivo* inhibition by this drug on the incorporation of preformed adenine into DNA adenine(8). This may help to explain, in part, the mechanism by which β-D-arabinosylcytosine exerts toxicity on the bone marrow(4) since bone marrow is dependent on supplies of preformed purines(9). No explanation is readily available for the paradoxical effects of this drug on adenylate reductase, *i.e.*, the reversal of inhibition at higher concentrations. This reversal effect may or may not relate to events that occur *in vivo*.

The stimulation (positive modulation) of guanylate reductase by deoxythioguanylate provides the first straightforward evidence that the mammalian enzyme is an allosteric protein—that the mammalian guanylate reductase is a regulatory enzyme. These data also suggest that only very small changes in molecular structure of the modulator suffice to cause the conformational change in the enzyme leading from inhibition to stimulation. The covalent radius of oxygen is 0.74 Å and that of sulfur is 1.04 Å. If DNA synthesis is dependent on a critically balanced supply of

TABLE VI. Effects of Various dTGMP and dGMP Concentrations on Guanylate Reductase.

Drug concentration	μμmoles dGDP formed: Exp I dTGMP added	Exp II dGMP added
0	37	52
5×10^{-6} M	58 (+57)	27 (−48)
10^{-5} M	54 (+46)	17 (−67)
5×10^{-5} M	54 (+46)	4 (−92)
10^{-4} M	49 (+32)	2 (−96)

Each vessel contained GDP-8-^{14}C, 15 mμmoles; ATP, 0.25 μmoles; Tris-HCl, pH 7.4, 5.0 μmoles; $MgCl_2$, 2.5 μmoles; enzyme extract, 3.1 mg protein; in a final volume of 0.51 ml. Incubations were carried out for 10 min. Numbers in parentheses refer to percent stimulation (+) or inhibition (−).

proximal precursors, stimulation of reductase activities by antimetabolites could be as damaging to cellular metabolism as inhibition.

The results of the study presented here reinforce the conclusions reached by Larsson and Reichard(1), Moore and Hurlbert(2), and others(3,8) that inhibitors and stimulators of ribonucleotide reductases control the supply of proximal precursors for DNA synthesis and, ultimately, cellular replication.

Summary. The purine ribonucleotide reductases from a mouse tumor were studied *in vitro*. Adenylate and guanylate reductions were subject to end-product inhibition. β-D-arabinosylcytosine produced paradoxical inhibitory effects on deoxyadenylate biosynthesis. The adenylate reductase was stimulated by dGTP and guanylate reductase was stimulated by β-D-deoxythioguanylate.

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Influence of Phenylbutazone on Gastric Secretion of Mucus.* (32288)

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Gastrointestinal symptoms often complicate the therapeutic use of phenylbutazone. It has been estimated that at least 2% of patients receiving the drug develop a peptic ulcer or experience recurrence of a previously occurring ulcer(1). The onset of gastrointestinal bleeding with or without formation of a peptic ulcer occurs more often(2). Administration of phenylbutazone to animals causes ulcers in guinea pigs(3), dogs(4), and rats(5). Other effects are gastritis and mucosal hemorrhage. The pathophysiology of gastric mucosal injury by phenylbutazone remains unknown. In dogs(6), rats(5) and guinea pigs(7), phenylbutazone lowers gastric acid, thus excluding the possibility that its effect on the stomach is mediated by hyperacidity. One cannot invoke a local, necrotizing effect of the drug on the gastric mucosa, because gastric changes are as intense after parenteral as after oral administration of the drug(8).

It has been postulated that the layer of mucus secreted by mucus-producing cells of the mucosa, and in particular by the prepyloric glands, protects the underlying mucosa by preventing acid-peptic secretions from gaining free access to it. Previous studies from this laboratory have shown that compounds such as cortisone(9) and aspirin(10), which have antirheumatic properties similar to those of phenylbutazone and also cause gastric mucosal injury, diminish the secretion of mucus by the canine gastric mucosa. The purpose of the studies forming the basis for this report was to determine if phenylbutazone interferes with secretion of gastric mucus in a way that may explain the gastric mucosal injury that it occasionally causes.

Materials and methods. Vagally denervated gastric-antral pouches were constructed in 4 dogs weighing from 16 to 30 kg. Care was taken to exclude acid secreting mucosa from the pouches. Secretions were drained *via* a

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