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Comparison of the Ribonucleotide with the Canavanine Reductase System (37916)

ARNOLD SCHULTZ, MASAHIDE SASAKI,[1] AND SAMUEL NATELSON
Department of Biochemistry, Michael Reese Medical Center, Chicago, Illinois 60616

Canavanine and canavaninosuccinate may be cleaved by reduction to form guanidine and guanidinosuccinate, respectively, and homoserine. This system requires reduced lipoate as the hydrogen donor. Dithiothreitol (Cleland's reagent) may substitute for the reduced lipoate, but other hydrogen donors such as NADH, NADPH, and reduced glutathione (GSH) may not be substituted for the lipoate. This system requires ferrous ion; other metal ions may not be substituted. Transferrin, in amounts equivalent to the Fe^{2+} content of the system, will completely inhibit it. An interesting observation with this system is the effect of 2,3-dimercapto-1-propanol (BAL) in acting as a potent inhibitor. Thus, this system which requries a hydrogen donor with thiol groups on alternate carbons such as reduced lipoate is totally blocked by the vicinal dithiol compound BAL (1, 2).

In the ribonucleotide reductase system, reduced lipoate or dithiothreitol serves as hydrogen donor. This system is activated by ferrous ion (3–5). Since there are similarities in the specific cofactors required by the ribonucleotide reductase system and the system which reduces canavanine and canavaninosuccinate, the effect of the presence of these guanidino derivatives on the ribonucleotide reductase system was studied. The effect of the inhibitors of the canavanine reductase system on the ribonucleotide reductase system were also explored.

Materials and Methods. Method of assay. Ribonucleotide reductase was prepared by a method previously described by Moore (6). The activity of the preparation was 0.68 nmoles/mg protein/min. This activity was observed without added Fe^{2+}.

The method of measurement of ribonucleotide reductase activity followed closely a method previously described (7). It was modified to test the various additives without change in concentration of the major components. The final volume in each tube was 120 μl. This comprised 20 μl of enzyme solution, 25 μl of substrate, varying volumes of inhibitor solution and water to make a final volume of 120 μl. The concentration of the various components in the 25 μl of substrate added was ATP 9 m*M*; dithiothreitol 0.03 *M*; Mg^{2+} 0.02 *M*; CDP-C^{14} 0.8 m*M*, 0.05 μCi/ml; phosphate buffer pH 7.0, 0.04 *M*.

When thiols, other than dithiothreitol, were evaluated as potential hydrogen donors, the dithiothreitol was omitted. Reduced lipoate (0.1 *M*) was prepared in 0.2 *M* Tris buffer, pH 7.3. Five or ten microliters were added to each tube to attain the concentration desired when the mix was diluted to a final volume of 120 μl with water. For BAL, cysteine, and reduced glutathione (GSH), 0.1 *M* solutions were prepared in water, adjusting the pH of the solutions to 7.0 with 1 *N* NaOH solution.

The solutions were incubated for 30 min at 37°. The reaction was stopped by heating at 100° for 3 min, and the mix was cooled in an ice bath. Alkaline phosphatase (30 μl) (EC 3.1.3.1, from *E. coli*, Worthington) diluted to 70 U/ml with Tris buffer (0.5 *M*, pH 8.0) were added, and the mix

[1] Present address: Kawasaki Medical School, Hospital, 2-1-80, Nakasange, Okayama, Japan.

was incubated at 37° for 30 min. The solution was again heated at 100° for 3 min to stop the reaction, cooled to room temperature, and centrifuged for 10 min at 20,000 rpm in an International #1 centrifuge. The supernatant was chromatographed to determine the amount of deoxycytidine formed (8).

Twenty microliters of a carrier solution, 10 m*M* with respect to each of cytidine, cytosine, and deoxycytidine, were spotted in a series of spots on Whatman No. 1 chromatography paper (46 × 57 cm) and allowed to dry. The supernatants (50 μl) from the different experiments were spotted on all of the dried carrier solution spots, except one which was the control. The compounds were resolved in 15 hr by descending paper chromatography using a 5 *M*, pH 9.5, ammonium acetate buffer, saturated borax, 0.4 *M* EDTA, and ethanol solution in the ratio of 10:40:0.3:110. The paper was dried and the deoxycytidine located by irradiation with an uv lamp. R_f values for deoxycytidine, cytidine, and cytosine were 0.88, 0.59, and 0.76, respectively.

The cytidine and deoxycytidine spots were located from the control, cut out, and placed in 15 ml of a 10% naphthalene solution in dioxane containing 0.7% PPO and 0.03% POPOP for counting. Spots cut out at the origin were also counted. To determine the original radioactivity, 25 μl of substrate were added to 15 ml of the scintillation mix. The samples were counted in the Packard Tri-Carb scintillation spectrometer.

Reagents. The BAL, cysteine, GSH, reduced lipoate, and hydroxurea were obtained from Sigma Chemical Co. The dithiothreitol was from Calbiochem. The ethylenediamine tetraacetic acid (EDTA) was reagent grade (Matheson). All of these solutions were made 0.1 *M*, the pH being adjusted to 7.0 with 4 *M* KOH if necessary.

Ferrous ion was added when required from a 0.1 *M* solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Mohrs' salt). Magnesium was added as a 0.1 *M* solution of magnesium acetate in water. Adenosine triphosphate was used as a 0.2 *M* solution in water of the sodium salt (Sigma), adjusting the pH to 7.0 with 4 *M* KOH.

A 0.1 *M* canavanine solution was prepared by dissolving 274 mg of canavanine sulfate (Calbiochem) in 4 ml of water and adding saturated $Ba(OH)_2$ drop by drop to pH 7.0 (2–2.5 ml). The solution was made to 10 ml and centrifuged to remove the sulfate. The supernatant was used.

A 0.1 *M* canavaninosuccinate solution, pH 7.0, was prepared by dissolving the barium salt, prepared enzymatically, and adding saturated sodium sulfate to remove the barium by centrifugation (2).

The amount of deoxycytidine formed increased with increasing amounts of dithiothreitol until a concentration of 6.25 mmoles/liter was reached. This concentration of dithiothreitol was therefore used in our experiments.

Effect of BAL on hepatoma cell proliferation. Female Sprague–Dawley (SPD) rats weighing from 140 to 180 g were used in the experiment. BAL dissolved in peanut oil was injected subcutaneously and for 8 days thereafter. Dosage was 30 and 60 mg/kg. On the second day, 1.5 ml of a suspension of Novikoff hepatoma cells from the peritoneal cavity of a rat was injected in each intraperitoneally. The cell count in the hepatoma cell suspension varied from 48,000 to 53,000 cells/cm during the various experiments. The culture was maintained by transplant from rat to rat after 8 days of incubation. The rats were autopsied and sections made of kidney, spleen, sternum, and liver. Smears on the ascites fluid were also examined.

Results. Table I compares the percentage of inhibition of the ribonucleotide reductase system by the various substances tested, at 4.17 m*M* concentration. From Table I, it will be noted that canavanine, glutathione, and cysteine do not seem to have any significant effect on the system. Canavaninosuccinate and lipoate can be seen to be moderate inhibitors. 2,3-Dimercaptopropanol (BAL), ethylenediamine tetraacetic acid (EDTA), and hydroxyurea are all strong inhibitors of this system.

Table II summarizes the data obtained when the thiol derivatives lipoate, BAL, glutathione, and cysteine were studied as substitutes for dithiothreitol in the ribonu-

TABLE I. Effect of Various Substances on Ribonucleotide Reductase Activity.[a]

Compound	Activity (%)	Compound	Activity (%)
Hydroxyurea	6.5	Canavaninosuccinate	77.5
EDTA	7.9	Cysteine	95.7
BAL	10.0	Homoserine	99.1
Hydroxylamine	28.9	Canavanine	100.5
Transferrin	56.3	Glutathione (reduced)	107.3
Lipoate (reduced)	56.5		

[a] Concentration was 4.17 mmoles/liter, except for transferrin which was 47 μmoles/liter. Dithiothreitol was 6.25 mmoles/liter in each case.

TABLE II. Effect of Various Thiol Derivatives as Hydrogen Donors in the Ribonucleotide Reductase System.[a]

Thiol derivative	Concentration (m*M*)	Deoxycytidine produced (nmoles)
Dithiothreitol	12.50	2.51
(Cleland's Reagent)	6.25	2.18
	4.17	1.98
Lipoate (reduced)	8.33	1.07
	4.17	1.06
2,3-Dimercapto-1-propanol (BAL)	8.33	0.20
	4.17	0.18
Cysteine	8.33	0.18
	4.17	0.14
Glutathione (reduced)	8.33	0.09
	4.17	0.06
Control	None	0.04

[a] Incubation time 30 min.

cleotide reductase system. Only dithiothreitol and lipoate demonstrate significant activity as hydrogen donors in this reaction. Dithiothreitol is approximately twice as active as lipoate in this regard.

Figure 1 illustrates the effect of added ferrous ion on counteracting the inhibitory effect of BAL on the system studied. The concentration of dithiothreitol in this experiment in each tube was 6.25 m*M*. The concentration of BAL used was 0.8 m*M* at which level it reduces activity to 50% of the control. Varying amounts of ferrous ion were then added. It is to be noted that only 50% of the inhibitory action of BAL was removed at a ferrous ion concentration of 0.6 mmoles/liter. At ferrous ion concentrations greater than this, inhibition was observed.

In the control tubes for this experiment, 2.1 nmoles of deoxycytidine were formed in each tube when neither BAL nor Fe^{2+} was added. Addition of Fe^{2+} in the control tube had no effect until a concentration of 40

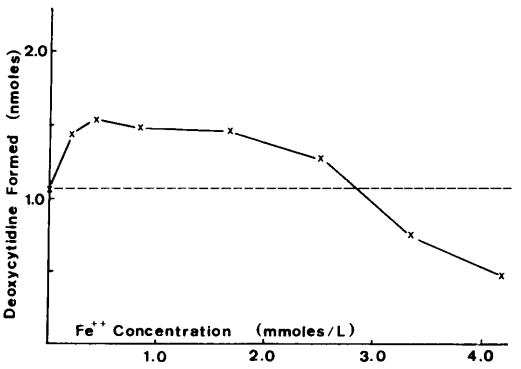

FIG. 1. Effectiveness of Fe^{2+} in removing the inhibition of BAL on the ribonucleotide system.

nmoles/liter was reached when production of deoxycytidine was lowered to 1.9 nmoles.

The experiments on the effect of BAL on the proliferation of hepatoma cells in the rat showed no significant difference between the controls and those at either level at which BAL was administered. No significant difference was noted as to the extent of infiltration of the tissues. Table III summarizes the data obtained in these experiments.

Discussion. Canavanine is toxic to mammals and certain other organisms. This toxicity is attributed, generally, to its resemblance to arginine and its substitution in protein synthesis.

Since both the canavanine and the ribonucleotide reductase systems require Fe^{2+} and lipoate for their activity, it was felt that some of the canavanine's toxicity might be due to its inhibition of the ribonucleotide reductase system, especially since canavanine interferes with DNA synthesis. Reduced lipoate or dithiothreitol serves to reduce thioredoxin in the ribonucleotide system (9).

As can be seen from Table I, canavanine has no effect on the ribonucleotide reductase system. The effect of canavaninosuccinate in producing partial inhibition may be attributed to its structure which is conducive to chelate formation with Fe^{2+}.

The total concentration of Fe^{2+} in the reaction mixture was analyzed, after digestion, and was found to be 5.6 μmoles/liter. Transferrin was added in increasing amounts up to 47 μmoles/liter or approximately 8 times the total Fe^{2+} concentration, yet only 50% inhibition could be reached. This should be contrasted with the fact that amounts of transferrin, equimolar to the free Fe^{2+} concentration, will totally inhibit the canavanine reductase system (2). This suggests that Fe^{2+} is not an absolute requirement for the ribonucleotide reductase system.

TABLE III. Fate of Rats to Whom BAL was Administered.[a]

Dosage	Number in group	Number with hepatoma	Survived (9 days)
Control (none)	15	15	15
30 mg/kg	15	12	15
60 mg/kg	15	12	13

[a] All rats received hepatoma cells intraperitoneally.

A significant finding was that 2,3 dimercapto-1-propanol (BAL) was a strong inhibitor of the ribonucleotide reductase system. This had not been observed before. The system was set up with BAL in a concentration (0.8 mmoles/liter) so as to reduce activity by 50%. Increasing amounts of Fe^{2+} were added to attempt to remove this inhibition, since BAL forms a complex with Fe^{2+}. Figure 1 suggests that the inhibitory effect of BAL is probably not due solely to its sequestering of the ferrous ion.

The observation that BAL was a potent inhibitor of the ribonucleotide system suggested the possible utilization of this phenomenon for inhibition of neoplasm growth. The inhibitor hydroxyurea has been used in the treatment of certain tumors (10). A literature search revealed that some preliminary studies on survival time had been made with BAL in the National Cancer Inst. Screening Program (11). These tests were negative. However, in view of the importance of the problem, we tested BAL against the Novikoff hepatoma in the rats we were using as a source of this enzyme. We included in our study histological and cytological examination of various tissues. Dosage was at a level approximately that which has been used for BAL in humans in cases of lead poisoning, and twice that level. We also started the injections 24 hr before inoculating with the hepatoma cells to see whether BAL would interfere with the implantation and growth of the hepatoma cells.

No evidence was found that BAL interfered with implantation of the tumor cells, their growth, their infiltration into tissues including bone marrow, or the well-being or length of survival of the animals. It is conceivable that the reason for this lack of success is the fact that BAL as such may be metabolized or excreted before it reaches the target enzyme system. This needs further investigation.

Summary. Reduced lipoate and dithiothreitol act as hydrogen donors in the canavanine and ribonucleotide reductase systems. Other thiol derivatives such as cysteine, 2,3-dimercapto-1-propanol (BAL), and glutathione cannot substitute effectively for the lipoate or dithiothreitol. Fe^{2+} acts as a cofactor for both systems. The canavanine system is inhibited quantitatively by transferrin or BAL. Transferrin in sixfold excess will only inhibit the ribonucleotide reductase system by approximately 50%. BAL is a strong inhibitor of the ribonucleotide reductase system. Addition of excess Fe^{2+} does not remove all of the inhibition produced by BAL in the ribonucleotide reductase system. BAL administered subcutaneously to rats before and after intraperitoneal inoculation with hepatoma cells did not prevent the proliferation of the hepatoma cells.

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