EFFECT OF HYDROXYUREA ON RIBONUCLEOTIDE REDUCTASE

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Hydroxyurea, an antineoplastic agent, inhibits DNA synthesis and cell proliferation without affecting RNA and protein synthesis (Gale, 1964; Young and Hodas, 1964; Yarbro *et al.*, 1965; Rosenbranz and Levy, 1965). A variety of evidence, primarily *in vivo*, has accumulated which suggests that the mode of action of hydroxyurea is the inhibition of the conversion of ribonucleotides to deoxyribonucleotides, a critical step in DNA synthesis (Frenkel *et al.*, 1964; Adams and Lindsay, 1967; Frenkel and Arthur, 1967; Elford, 1967).

It was decided to investigate the direct effect of hydroxyurea on a completely *in vitro* ribonucleotide reduction system isolated from three of the most thoroughly studied deoxynucleotide synthesizing systems, *Escherichia coli*, *Lactobacillus leichmannii*, and Novikoff hepatoma rat tumor. In addition, the effect of hydroxyurea on several animal ribonucleotide reduction systems was studied.

The data from this study support the proposition that hydroxyurea inhibits DNA synthesis by interfering with ribonucleotide reduction in various animal systems and in *E. coli*, but has no effect on the reduction system of *L. leichmannii*.

MATERIALS AND METHODS

The Novikoff tumor was prepared in the following manner: When the tumor reached the size of 5 to 8 gms it was removed, freed from necrotic material and extracted in a Potter-Elvehjem homogenizer. The extract was centrifuged at 25,000 x g, and the supernatant was treated with protamine sulfate and fractionated with ammonium sulfate to further purify the enzyme. The other animal systems were prepared in a similar manner.

E. coli 70V3-462[1] a thymine mutant was grown under the derepressed conditions of Biswas *et al.*, (1965), and *E. coli* B harvested one quarter into the exponential phase of growth while *L. leichmannii* 7830 was grown on limiting cyanocobalamin (Ghambeer and Blakley, 1965). The bacterial extracts were prepared by alumina grinding and centrifugation at 25,000 x g.

Hydroxyurea and dihydroxyurea were purchased from Nutrional Biochemical Co. Unlabeled nucleotides were obtained from P-L Biochemicals and Calbiochem; Radioactive ^{3}H-CDP and ^{3}H-CTP were purchased from Schwarz Bioresearch. The reduction of ribonucleotides to deoxynucleotides was measured by monitoring the conversion of cytosine ribonucleotide to the deoxy compound utilizing a slightly modified assay procedure developed originally by Reichard *et al.*, 1961. One enzyme unit is defined as the formation of one hundred $\mu\mu$ moles of deoxynucleotide per hour.

RESULTS AND DISCUSSION

The results in table 1 illustrate the effect of hydroxyurea on the ability of crude extracts of *E. coli*, *L. leichmannii* 7830 and Novikoff tumor to catalyze the reduction of ribonucleotides to deoxynucleotides. Hydroxyurea inhibited CDP reduction in *E. coli* and the Novikoff hepatoma tumor extract, but had no effect on the reduction of CTP by *L. leichmannii* even at a concentration of 10^{-3}*M*. The results were more consistent with the tumor extracts than with the *E. coli* preparations.

The effect of hydroxyurea on ribonucleotide reduction was reassayed utilizing a 25-fold purified enzyme preparation of the Novikoff tumor. The data (Figure 1) show that this purified material exhibits an increased susceptibility to hydroxyurea. There is an 80% inhibition with 10^{-3}*M* hydroxyurea which abruptly diminished with decreasing concentrations of hydroxyurea. The level at which hydroxyurea inhibits ribonucleotide reduction *in vitro* is slightly higher than that required for the impairment of several *in vivo* processes such as thymine incorporation into DNA in Hela cells (Young *et al.*, 1967) and transformed BHK-21 cells (Pollak and Rosenkranz, 1967).

The preceeding results were obtained employing dithiothreitol because the 25-fold partially purified ribonucleotide reductase preparation is devoid of activity without the addition of a reducing substance. Dithiothreitol serves as a replacement or supplement for the natural physiological

[1] Gift of Dr. T. R. Breitman of N.I.H.

TABLE 1

Effect of Hydroxyurea on Ribonucleotide Reductase in Crude Extracts

	Novikoff		*E. coli*		*L. leichmannii*	
Hydroxyurea molarity	Reductase units	Inhibition %	Reductase units	Inhibition %	Reductase units	Inhibition %
0	5.3	-	88.6	-	415	-
10^{-6}	5.4	0	91.0	0	428	0
10^{-5}	4.8	8	87.0	2	433	0
10^{-4}	3.9	27	68.5	23	397	2
10^{-3}	1.6	70	35.5	60	410	0

Incubation vessels contained: 0.120 μ mole ribonucleotide ribonucleotide-H^3 1.13 μ moles ATP, 0.5 μ mole $MgCl_2$, 3 μ moles K phosphate buffer pH 7.4, 5 μ moles dithiothreitol in 340 μl. Incubation was for 60 min. at 370. The *L. leichmannii* reaction mixture also contained 5 mμ moles of coenzyme B_{12}.

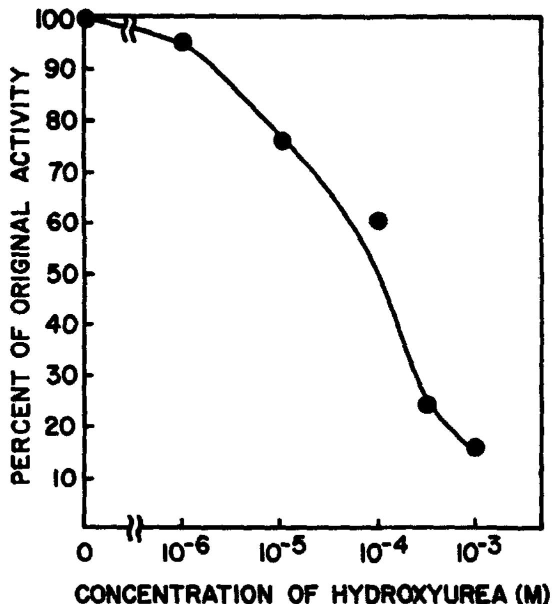

Figure 1. The Effect on Hydroxyurea on Purified Tumor Ribonucleotide Reductase.

TABLE 2

Effect of Hydroxyurea on Purified Tumor Ribonucleotide Reductase with Physiological Reducing System

Hydroxyurea	Reductase	Inhibition
molarity	units	%
0	54.5	-
10^{-6}	51.8	5
10^{-5}	44.5	18
10^{-4}	16.7	69
10^{-3}	3.2	94

The conditions of assay were as described in table 1 except that 0.2 µ mole of NADPH and 1 mg of the Novikoff tumor reducing complex were included.

reducing system which is presumed to be similar to the thioredoxin-thioredoxin reductase system of *E. coli* (Lauerent *et al.*, 1964; Moore *et al.*, 1964) and *L. leichmannii* (Orr and Vitols, 1966).

During the purification of the ribonucleotide reductase a second fraction was separated from the ribonucleotide reductase. This second component appears to have properties and functions analogous to the thioredoxin-thioredoxin reductase systems. When this complex is added to the purified ribonucleotide reductase, activity is restored in the absence of dithiothreitol.

Because of the possibility that an interaction between hydroxyurea and dithiothreitol was the cause of inhibition, hydroxyurea was tested on a ribonucleotide reduction system utilizing the physiological reducing system devoid of dithiothreitol. However, the data (table 2) obtained with the tumor reduction system is in agreement with the chemical reducing system.

The specificity of hydroxyurea inhibition was examined in a limited way by comparing the effect of urea and dihydroxyurea. The results showed that urea had no discernable effect while dihydroxyurea, a compound reported by Young *et al.*, (1967a) to have a slightly greater impact than hydroxyurea on incorporation of thymine into DNA in Hela cells, was a more potent inhibitor than hydroxyurea in the *in vitro* system.

TABLE 3

Effect of Hydroxyurea on Ribonucleotide Reductase in Animal Extracts

	Morris Tumor 3924A		Chick Embryo		LM Cells		Human Melanoma	
Hydroxyurea	Reductase Activity	Inhibition	Reductase Activity	Inhibition	Reductase Activity	Inhibition	Reductase Activity	Inhibition
molarity	units	%	units	%	units	%	units	%
0	15.2	-	10.3	-	34.3	-	1.2	-
10^{-3}	5.64	63	3.8	54	3.89	89	0.75	41
10^{-4}	12.1	21	6.7	30	29.5	12	0.95	25
10^{-5}	12.9	15			36.0	0	1.12	10

The reaction mixture contained the components as listed in table 1.

A survey (table 3) of the effect of hydroxyurea on several animal *in vitro* ribonucleotide reduction systems was undertaken because of the ambivalent results obtained with the two microbial systems. Experiments with extracts of rat hepatoma of the Morris line 3924A, mouse fibroblast (LM) cells, five day chick embryo and human melanoma are included in the data. In all cases there was inhibition of ribonucleotide reduction at 10^{-3}*M* hydroxyurea. Since hydroxyurea has proven useful in the treatment of melanoma (Lerner *et al.*, 1965; Cole *et al.*, 1965), it is of special interest to note that hydroxyurea inhibited the ribonucleotide reduction of a melanoma extract.

The effect of hydroxyurea may be related to the requirement for vitamin B_{12} supplementation. Ribonucleotide reduction systems which do not require additional vitamin B_{12}, *i.e.* *E. coli* and animal systems, are inhibited while *L. leichmannii*, which does require the addition of coenzyme B_{12}, is not affected.

This paper provides evidence that hydroxyurea prevents DNA synthesis in animal systems by inhibiting ribonucleotide reduction. The data do not restrict the mode of action of hydroxyurea to this metabolically important event but supports the contention that it must be one of the major effects, since no other reaction in the sequence leading to DNA synthesis has been shown to be affected by hydroxyurea. The experiments of Yarbro (1968) and Pollak and Rosenkranz (1967) in which deoxynucleosides added individually or in combination failed to overcome the effect of hydroxyurea does not negate the proposition that hydroxyurea interferes with ribonucleotide reduction. The complexity of the regulatory mechanisms of this reaction (Reichard, 1968) requires a fortuitous balance of nucleotides. Such appears to be the case in the work of Young *et al.*, (1967b), and Adams and Lindsay (1967) where the addition of deoxynucleosides corrected the hydroxyurea impairment.

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REFERENCES

1. Adams, R., L., and Lindsay, J. G., J. Biol. Chem. 242, 1314 (1967).
2. Biswas, C., Hardy, J., and Beck, W. S., J. Biol. Chem. 240, 3631 (1965).
3. Cole, D. R., Beckloff, G. L., and Rousselot, L. M., N. Y. State J. Med. 65, 2132 (1965).
4. Elford, H. L., Federation Proc. 26, 838 (1967).
5. Frenkel, E. P., Skinner, W. N., and Smiley, J. D., Cancer Chemotherapy Rept. 40, 19 (1964).

6. Frenkel, E. P., and Arthur, C., Cancer Res. 27, 1016 (1967).
7. Gale, G. R., Biochem. Pharmacol. 13, 1377 (1964).
8. Ghambeer, R. K., and Blakley, R. L., Biochem. Biophys. Res. Commun. 21, 40 (1965).
9. Laurent, T. C., Moore, E. C., and Reichard, P., J. Biol. Chem. 239, 3436 (1964).
10. Lerner, H. J., and Beckloff, G. L., J. Amer. Med. Assoc. 192, 1168 (1965).
11. Moore, E. C., Reichard, P., and Thelander, P., J. Biol. Chem. 239, 3445 (1964).
12. Orr, M. D. and Vitols, E., Biochem. Biophys. Res. Commun. 25, 109 (1966).
13. Pollak, R. D., and Rosenkranz, H. S., Cancer Res. 27, 1214 (1967).
14. Reichard, P., Baldesten, A., and Rutberg, L., J. Biol. Chem. 236, 1150 (1961).
15. Reichard, P., European J. Biochem 3, 259 (1968).
16. Rosenkranz, H. S., and Levy, J. A., Biochim. Biophys. Acta 95, 181 (1965).
17. Yarbro, J. W., Kennedy, B. J., and Barnum, C. P., Proc. Natl. Acad. Sci. 53, 1033 (1965).
18. Yarbro, J. W., Cancer Res. 28, 1082 (1968).
19. Young, C. W. and Hodas, S., Science 146, 1173 (1964).
20. Young, C. W., Schochetman, G., Hodas, S., and Balis, M. E., Cancer Res. 27, 535 (1967a).
21. Young, C. W., Schoochetman, G., and Karnofsky, D. A., Cancer Res. 27, 525 (1967b).