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The Effect of Deoxyribonucleoside and Ribonucleoside Addition on the Inhibitory Effect of Hydroxyurea on Deoxyribonucleic Acid Synthesis

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Hydroxyurea has been demonstrated to be a potent inhibitor of DNA synthesis, with little or no immediate effect on RNA synthesis, in numerous systems (Yarbro, Kennedy & Barnum, 1965; Yarbro, Niehaus & Barnum, 1965b; Young & Hodas, 1964; Schwartz, Garofalo, Sternberg & Philips, 1965). It has been shown not to influence synthesis of protein in several systems (Yarbro *et al.* 1965b; Young & Hodas, 1964); however, it has been demonstrated to inhibit the synthesis of histone (Yarbro *et al.* 1965b), especially lysine-rich histone (Yarbro, 1966). The mechanism of action of hydroxyurea in producing inhibition of DNA synthesis has not been fully elucidated, although it has been suggested that this agent blocks the conversion of ribonucleotides into deoxyribonucleotides (Frenkel, Skinner & Smiley, 1964), that it has a direct action producing cleavage of DNA (Borenfreund, Krim & Bendich, 1964), or that it inhibits pyrimidine synthesis (Vogler, Bain & Huguley, 1965). A number of agents of similar structure have been shown to inhibit DNA synthesis. These include hydroxyurethane, dihydroxyurea, hydroxy-*N*-methylurea, *N*-ethylhydroxyurea, *N*-hydroxyguanidine, *N*-methylacetohydroxamic acid, *N*-methylhydroxylamine and acetohydroxamic acid (Young, Schochetman, Hodas &

Balis, 1967b). Young, Schochetman & Karnasky (1967a) have shown that addition of deoxyribonucleosides partially reverses the inhibitory action of hydroxyurea. Mohler (1964) has shown a similar effect in the Chinese-hamster-cell culture system.

Because of the selective nature of the action of this agent against DNA synthesis, and particularly because it appears to inhibit histone synthesis simultaneously, the nature of its inhibitory action was investigated further.

The system used for the study was 6C3HED mouse ascites tumour. Hydroxyurea in a dose of 60mg./kg. body wt. was administered by intraperitoneal injection in 0.9% NaCl, and control animals received an equal volume of 0.9% NaCl. Immediately thereafter, 100 μ C of sodium [32 P]-phosphate was given by intraperitoneal injection. At the time of administration of hydroxyurea, a portion of the animals received a mixture of the four deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) in a concentration such that each mouse received 2.0 μ moles of each deoxyribonucleoside. Other animals received a mixture of the ribonucleosides (adenosine, guanosine, cytidine and uridine) in a similar fashion such that each mouse received 1.2 μ moles of each ribonucleoside. The animals

Table 1. *Effect of hydroxyurea, ribonucleosides and deoxyribonucleosides on the incorporation of [32 P]-phosphate into RNA and DNA of 6C3HED mouse ascites tumour*

Incorporation of [32 P]phosphate into the nucleic acids of 6C3HED mouse ascites tumour was evaluated after incubation *in vivo* for 1 hr. with and without injection of hydroxyurea, ribonucleosides and deoxyribonucleosides. The relative specific activities are given as means $\pm$ s.d. of the results for groups of five to 15 mice.

	RNA		DNA	
	Relative sp. activity	% of control	Relative sp. activity	% of control
Control	3.91 $\pm$ 1.19	100	1.05 $\pm$ 0.379	100
Hydroxyurea	3.73 $\pm$ 0.893	95	0.396 $\pm$ 0.0929	38
Ribonucleosides	6.10 $\pm$ 0.966	156	1.75 $\pm$ 0.380	167
Ribonucleosides + hydroxyurea	4.88 $\pm$ 1.38	125	0.324 $\pm$ 0.0842	31
Deoxyribonucleosides	4.38 $\pm$ 0.712	112	1.31 $\pm$ 0.203	125
Deoxyribonucleosides + hydroxyurea	3.83 $\pm$ 0.551	98	0.453 $\pm$ 0.0602	43

were killed 1 hr. after the administration of the [32 P]phosphate, the ascites-tumour fluid was drained into tubes in an ice bath, and an equal volume of 5% (w/v) trichloroacetic acid was added, after which the tumours were homogenized. DNA and RNA were isolated and their specific activity determined by the methods described by Yarbro *et al.* (1965a). DNA and RNA specific activities (counts/min./ μ g. of nucleic acid phosphorus) were expressed as percentages of the specific activity of the acid-soluble phosphorus from each sample as described by Yarbro *et al.* (1965a), and this was termed relative specific activity.

Table 1 illustrates the means $\pm$ s.d. obtained from the various groups of animals studied. Hydroxyurea produced an inhibition of DNA synthesis to 38% of control values, whereas RNA synthesis was 95% of control. This is in accord with previously reported observations. Addition of ribonucleosides to the system resulted in a stimulation of incorporation of [32 P]phosphate into both RNA and DNA to values of 156% and 167% of the control respectively. Hydroxyurea, in combination with the four ribonucleosides, produced inhibition of the ribonucleoside stimulation of DNA synthesis (31% of the control), but did not prevent ribonucleoside stimulation of RNA synthesis. This is in accord with the hypothesis that hydroxyurea blocks the conversion of ribonucleotides into deoxyribonucleotides. Addition of deoxyribonucleosides produced a stimulation of DNA but not of RNA synthesis. However, when hydroxyurea and deoxyribonucleosides were added simultaneously, the hydroxyurea-induced inhibition of DNA synthesis was not abolished.

If hydroxyurea has as its direct mechanism of action the inhibition of conversion of ribonucleotides into deoxyribonucleotides, then one might expect to see complete reversal of hydroxyurea inhibition by

the addition of deoxyribonucleosides. However, deoxyribonucleosides fail to reverse the hydroxyurea-induced inhibition of DNA synthesis. This suggests that hydroxyurea may have an alternative site of action. Vogler *et al.* (1965) have suggested that hydroxyurea may inhibit the synthetic pathway for pyrimidine at a stage before orotic acid formation in view of their observation that hydroxyurea reverses the oroticaciduria induced by azauridine in human subjects. However, our data are not consistent with such an alternative site of action since such inhibition should be corrected by added deoxyribonucleosides. Further, one would expect DNA and RNA to be equally inhibited by hydroxyurea, if such a mechanism were operative. The inhibition of the ribonucleoside stimulation of DNA synthesis by hydroxyurea is, of course, compatible with an action of hydroxyurea at the level of formation of deoxyribonucleotides, but is equally compatible with a site of inhibition of phosphorylation, or assembly of the deoxyribonucleotide triphosphates by DNA polymerase. However, Young & Hodas (1964) have reported that hydroxyurea does not inhibit either DNA polymerase or phosphorylation of the deoxyribonucleotide monophosphates.

It has been previously suggested that hydroxyurea does not exert the observed effects on DNA synthesis by direct attack on the DNA molecule after conversion into hydroxylamine because of the relative efficacy of hydroxylamine and hydroxyurea as inhibitors of DNA synthesis, the rapid action of hydroxyurea compared with that of hydroxylamine, and the fact that hydroxylamine inhibits RNA and protein synthesis in addition to DNA. However, this does not rule out the possibility of a secondary action of hydroxyurea directly against DNA. Such a double mechanism of action might explain the difficulty of reversing the hydroxyurea

effect by added deoxyribonucleosides. Alternatively, it has been postulated that the added deoxyribonucleosides are so rapidly cleaved to free bases as to be only partially effective in blocking the hydroxyurea effect (Young *et al.* 1967a). It seems unlikely, however, that all of the added deoxyribonucleoside was cleaved to the free base form, since the animals receiving the four deoxyribonucleosides but not hydroxyurea clearly showed a stimulation of the incorporation of phosphate into DNA that was significantly different from control values.

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The Effect of Aurovertin on a Soluble Mitochondrial Adenosine Triphosphatase

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A soluble ATPase* can be prepared from ox heart mitochondria by homogenizing a suspension of an acetone-dried powder of mitochondria in an all-glass homogenizer (Selwyn, 1962, 1967). The properties of this ATPase are similar to those of the coupling factor F_1 described by Pullman, Penefsky, Datta & Racker (1960) with respect to metal ion activator requirements, activation by 2,4-dinitrophenol, substrate specificity and cold lability. The present communication provides further evidence for their identity. Selwyn's (1967) ATPase preparation is shown to behave similarly to factor F_1 with respect to its interaction with factor CF_0 , the factor conferring oligomycin sensitivity on factor F_1 (Kagawa & Racker, 1966b). It is also shown that Selwyn's (1967) ATPase preparation is sensitive to aurovertin, a known inhibitor of oxidative phosphorylation and mitochondrial ATPase (Lardy, 1960; Kagawa & Racker, 1966a).

Methods. ATPase activity was measured by the method of Pullman *et al.* (1960). The soluble ATPase was preincubated at 30° for 3 min. in a solution containing 250 μ moles of sucrose, 25 μ moles of tris-sulphate buffer, pH 7.4, 0.3 μ mole of

NADH, 1 μ mole of KCN, 50 μ g. of lactate dehydrogenase and 25 μ g. of pyruvate kinase in a volume of 0.84 ml. To start the reaction, a prewarmed solution containing 2 μ moles of ATP, 2 μ moles of $MgSO_4$, 2 μ moles of phosphoenolpyruvate and 50 μ moles of tris-sulphate buffer, pH 7.4, in 0.16 ml. was added. The NADH oxidation was followed spectrophotometrically.

ATPase was prepared by the method of Selwyn (1967). It was purified to the stage of the second $(NH_4)_2SO_4$ fractionation, and stored in 50% glycerol at -20° until used. Factor CF_0 was prepared as described by Kagawa & Racker (1966b). Aurovertin was a generous gift from the Pitman-Moore Division of the Dow Chemical Co. (Zionsville, Ind., U.S.A.), and oligomycin A was given by Professor E. E. Van Tamelen. Oligomycin A and aurovertin were added to reactions as ethanolic solutions in volumes of 5 μ l. or less.

Results and discussion. The results of Expts. 1 and 5 (Table 1) show that the soluble ATPase is inhibited by factor CF_0 . Addition of phospholipid to the ATPase + factor CF_0 mixture restimulates the ATPase activity, and this activity is sensitive to oligomycin A (Expts. 6 and 7). The soluble ATPase by

* Abbreviation: ATPase, adenosine triphosphatase.