ACTIVATION OF DNA BIOSYNTHESIS IN EHRLICH ASCITES CELLS IN VIVO FOLLOWING APPLICATION OF 6-AZA-2′-DEOXYCYTIDINE

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It was found that deoxycytidylate deaminase from Ehrlich ascites cells is considerably activated by 6-aza-2′-deoxycytidine-5′-phosphate (Kára and Šorm, 1964). The activation of the enzyme by this synthetic nucleotide which was recently synthesized at the Institute of Organic Chemistry and Biochemistry in Prague (Pliml, Kára and Šorm, 1964) is probably effected through a feedback mechanism, the 6-aza-2′-deoxycytidine-5′-phosphate being bound at an allosteric site of the deoxycytidylate deaminase molecule and acting as the feedback activator of this enzyme. A similar feedback activation of deoxycytidylate deaminase by 2′-deoxycytidine-5′-triphosphate and a feedback inhibition of this enzyme by 2′-deoxythymidine-5′-triphosphate was described (Maley and Maley, 1962; Scarano et al., 1963).

It was reported earlier that 6-aza-2′-deoxycytidine-2-^{14}C is phosphorylated *in vitro* by soluble proteins from the Ehrlich ascites cells, giving rise to 6-aza-2′-deoxycytidine-5′-phosphate-2-^{14}C while 6-aza-2′-deoxyuridine-2-^{14}C is only little phosphorylated under identical conditions (Kára and Šorm, 1963a,b). We have observed in addition to this that the glycosidic bond of the 6-aza-2′-deoxycytidine molecule is metabolically stable and is not split *in vivo* after intra-

peritoneal application to mice (Kára, unpublished results). It could thus be assumed that 6-aza-2′-deoxycytidine can be phosphorylated by the Ehrlich ascites cells to 6-aza-2′-deoxycytidine-5′-phosphate which could activate the DNA biosynthesis through an interaction with deoxycytidylate deaminase in vivo. For this reason the incorporation of unspecifically labelled ^{14}C-cytidine into the DNA pyrimidine bases of the Ehrlich ascites cells in vivo was investigated and the quantitative changes of this incorporation with simultaneous i.p. application of 6-aza-2′-deoxycytidine followed. The results of this work are presented here.

Ehrlich ascites bearing mice (6th day after transplantation) were given i.p. ^{14}C-cytidine (commercial preparation of the Institute for Research, Production and Application of Radioisotopes in Prague) in two doses of 1.5 μC per mouse, the interval between the injections being 6 hours. In the group there were three animals. The second group of three mice was given ^{14}C-cytidine plus 5 μmoles 6-aza-2′-deoxycytidine in 0.2 ml. 0.9% sodium chloride solution per mouse (two doses as in the first group). The 6-aza-2′-deoxycytidine was synthesized by Dr.Pliml (Pliml and Šorm, 1963). The mice were killed 24 hours after the first injection, the tumour cells were centrifuged, washed three times with physiological saline and extracted with 10% sodium chloride solution containing 0.5% sodium dodecylsulfate for 1 min. at 90°C. The lysed cells were centrifuged at 16,000 r.p.m. for 45 min. and fibrous DNA from the supernatant was precipitated with two volumes of 96% ethanol. The isolated DNA was dissolved in 0.3M-NaOH and incubated at 37°C for 18 hours. The alkaline hydrolysis removed RNA and subsequent acidification with HCl resulted in a precipitation of DNA. Aliquots of the purified DNA solution were used for

estimating specific radioactivity of DNA in both groups. The DNA concentration in solution was estimated by the reaction with diphenylamine according to Burton (Burton, 1956).

Table 1 shows that the specific radioactivity of DNA in the second group (where 6-aza-2′-deoxycytidine was applied) is about twice as high as in the control group (by 97%).

Preparation investigated	Specific radioactivity (counts/min./μmole base, mg.DNA)		% Increase in specific radioactivity in group 2.
	1st group	2nd group	
^{14}C-DNA	6,072	11,925	97
^{14}C-thymine	1,131	1,924	70
^{14}C-cytosine	2,917	6,493	122

Table 1. Incorporation of ^{14}C-cytidine into DNA, specific radioactivity of DNA-thymine and cytosine in Ehrlich ascites cells (1st group) and increase of the incorporation of the radioactive precursor on simultaneous application of 6-aza-2′-deoxycytidine (2nd group).

The DNA aliquots were then hydrolyzed in 95% perchloric acid for 30 min. at 100°C, the hydrolyzates diluted with distilled water and adsorbed on a microcolumn of Norite, after washing the column with several ml. dest. H_2O the radioactive bases were eluted with 50% ethanol containing 5% ammonia. The eluted bases were chromatographically separated in isopropanol-HCl on Whatman paper No. 1 (Wyatt, 1951). The zones of thymine and cytosine were cut out, eluted and aliquots of the eluates used for spectrophotometric determination of the concentration of bases and for measuring the radioactivity on a flow counter. These values served for calculating the specific radioactivity of thymine and cytosine in DNA of both groups. No trace of uracil could be detected in the chromatogram of

the bases, this confirming the view that the preparation was completely free of RNA.

It follows from the results shown in Table 1 that the specific radioactivity of DNA-thymine is higher by 70% in the 2nd group of mice which were treated with 6-aza-2′-deoxycytidine. The distribution of radioactivity in DNA-cytosine and thymine in the control group 1 is in agreement with similar results of Prusoff (Prusoff, 1958). Specific radioactivity of DNA-cytosine was more than twice higher in the 2nd group. This significant increase of incorporation of the radioactive precursor into DNA-cytosine seems to indicate that 6-aza-2′-deoxycytidine-5′-phosphate could be involved in a feedback mechanism activating a number of enzymes anabolizing cytidine as is shown in Fig. 1 representing schematically the metabolism of pyrimidine nucleosides and nucleotides.

6-Aza-2′-deoxycytidine is very probably phosphorylated in the cell to the nucleotide 6-aza-2′-deoxycytidine-5′-phosphate (6-aza-dCMP) which activates deoxycytidylate deaminase (Kára and Šorm, 1964) and thus also the biosynthesis of deoxyuridylic acid and probably thymidine-5′-phosphates as precursors of DNA. At the same time, it seems possible that the enzymes synthesizing dCDP and dCTP could be activated, which is reflected in the significant increase in the incorporation of radioactive precursor into DNA-cytosine. Cytidine-5′-diphosphate reductase was described in Escherichia coli (Reichard et al.,1961) and similar enzymes reducing cytidine nucleotides probably on the level of triphosphates were found in the ascitic form of Novikoff's rat tumour (Moore and Hurlbert, 1962). The activation of these enzymes could enhance the utilisation of ^{14}C-cytidine for DNA biosynthesis. As is shown by our preliminary experiments the application of 6-aza-2′-deoxycytidine <u>in vivo</u> results also in

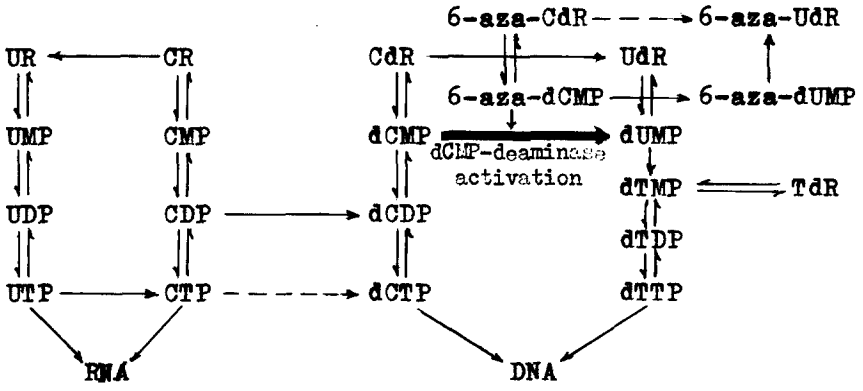

Fig. 1. Metabolism of cytidine (CR) and interaction with metabolites of 6-aza-2′-deoxycytidine (6-aza-CdR)

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a raised incorporation of ^{14}C-cytidine into RNA of Ehrlich ascites cells. The interpretation of the mechanism of action of 6-aza-2′-deoxycytidine on the nucleic acids biosynthesis *in vivo* requires of course an experimental investigation of a number of detailed mechanisms.

The results are in accord with the view that enzymes taking part in the biosynthesis of pyrimidine nucleotides are in a dynamic equilibrium in the living cell, the equilibrium being regulated by a feedback mechanism. Deoxycytidylate deaminase seems to play an important control role in this enzymatic sequence. It is of interest that 6-aza-2′-deoxycytidine-5′-phosphate is the only hitherto known synthetic analogue of the natural substrate which can act as a specific activator of deoxycytidylate deaminase (Kára and Sorm, 1964).

The activation of the ^{14}C-cytidine metabolism by 6-aza-2′-deoxycytidine *in vivo* might aid in pointing the way to other theoretically and practically important problems.

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