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THE ACTION OF SOME OROTIC ACID ANALOGUES ON THE *IN VITRO* INCORPORATION OF ^{14}C-OROTATE INTO PYRIMIDINE NUCLEOTIDES

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Abstract—The effect of some orotic acid analogues on the incorporation of ^{14}C-orotate into uridine and cytidine nucleotides by a soluble rat liver enzyme system was investigated. The compounds studied were found to inhibit the labelling of uridine nucleotides in the following order: 5-azaorotic acid, orotic acid hydrazide, 2-thioorotic acid, uracil-6-acetic acid, orotaldehyde hydrazone, 4, 6-dihydroxypicolinic acid ("3-deazaorotic acid"). With the orotic acid hydrazide and the orotaldehyde hydrazone the labelling of cytidine nucleotides runs parallel to that of uridine nucleotides. With the four other compounds the inhibition of ^{14}C-orotate incorporation into cytidine nucleotides is much stronger as compared to uridine nucleotides. This effect is more pronounced in the case of 2-thioorotic acid and uracil-6-acetic acid.

THE OROTIC ACID analogues are of particular interest as potential antimetabolites of nucleic acids biosynthesis. Some of these compounds, like 5-fluoroorotic acid and 5-azaorotic acid were found to have a pronounced inhibitory effect on the biosynthesis of pyrimidine nucleotides and nucleic acids *in vitro* and *in vivo*.[1, 2] Recently, a cell-free rat liver enzyme system was developed in this laboratory which allows the study of ^{14}C-orotate incorporation in both uridine and cytidine nucleotides.[3] We studied the inhibitory effect on this enzyme system of some pyrimidine analogues as listed in Fig. 1. These compounds were selected with the aim to have analogues with different changes in the molecule of orotic acid. The effect of 5-azaorotic acid on the *in vitro* biosynthesis of uridine nucleotides has been investigated previously by others.[2] There is no information in the literature concerning the effect of the other five compounds on the biosynthesis of pyrimidine nucleotides. Only scarce information exists

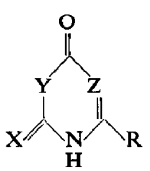

FIG. 1. 1. 5- Azaorotic acid (X = O, Y = NH, Z = N, R = COOH). 2. 4, 6-Dihydroxypicolinic acid ("3-Deazaorotic acid") (X = O, Y = CH_2, Z = CH, R = COOH). 3. 2-Thioorotic acid (X = S, Y = NH, Z = CH, R = COOH). 4. Orotic acid hydrazide (X = O, Y = NH, Z = CH, R = $CONHNH_2$) 5. Uracil-6-acetic acid (X = O, Y = NH, Z = CH, R = CH_2COOH). 6. Orotaldehyde hydrazone (X = O, Y = NH, Z = CH, R = $CHNNH_2$).

on their antimetabolic action. It has been reported that uracil-6-acetic acid does not inhibit the growth of *E. coli*.[4] 2-Thioorotic acid was reported to inhibit the mitotic cycle in chick embryo haemopoietic cells.[5] This compound was inactive towards OMP pyrophosphorylase at equimolar concentrations with the substrate, orotic acid.

METHODS AND MATERIALS

Male, albino rats, weighing from 150 to 200 g were sacrificed by decapitation and their livers removed immediately. The livers were homogenized in a Potter–Elvehjem glass-Teflon homogenizer with 2 volumes of a medium containing: 0·35 M sucrose, 0·004 M $MgCl_2$, 0·025 M KCl and 0·05 M Tris-HCl buffer (pH 7·6). The homogenate was filtered through a gauze and centrifuged at 105,000 *g* for 60 min in a MSE "Superspeed 40" refrigerated centrifuge. The clear supernatant was taken off carefully and used immediately or stored at −20°.

The reaction was carried out at 37° for 60 min with occasional shaking. The incubation mixture contained in a total volume of 2 ml: 1 ml of the liver supernatant as enzyme system, 6 μmoles ATP, 20 μmoles sodium phosphoenolpyruvate, 10 μmoles L-glutamine, 0·5 μmoles GTP, 40 μmoles $MgCl_2$ and 0·25 μmoles 6-^{14}C-orotate (14·6 mc/m-mole). The pyrimidine analogues studied were also added at concentrations as indicated in the text. Since aliquots of the same supernatant were used in all samples, the amount of endogenous 5-phosphoribosyl-1-pyrophosphate was the same and its addition unnecessary.

The reaction was stopped with 7 ml of 0·6 N $HClO_4$ and the precipitate was centrifuged down for 5 min at 6000 rpm. To the acid extract containing the free nucleotides 1μmole of UMP and 1 μmole of CMP were added as markers. The concentration of $HClO_4$ was adjusted to 1 N and the solution heated at 100° for 20 min. Under these conditions all free uridine and cytidine nucleotides are quantitatively converted into the respective monophosphates and fractionated further by ion-exchange chromatography with the subsequent use of Dowex 50-H^+ and Dowex 1-$HCOO^-$ columns according to the technique of Kammen and Hurlbert,[7] modified as follows. The perchloric acid extract was neutralized with KOH to pH 3·0 and kept for 1–2 hr at 4°. The $KClO_4$ was discarded and the supernatant passed through a 7 × 0·9 cm Dowex 50 column equilibrated with 0·001 N HCl. The fraction of UMP which is not retained by the column is collected and CMP further eluted with distilled H_2O. The UMP fraction is adsorbed after alkalization on a 6 × 0·9 cm column of Dowex 1. Contaminants are eluted with 0·1 M $HCOONH_4$ and then UMP with 0·3 M $HCOONH_4$.

The fractions UMP and CMP fractionated by the above technique are radiochemically pure. Samples of the two nucleotides were dried on planchettes and their radioactivity determined with a windowless gas-flow "Vakutronik" VA-Z-530 counter at about 40 per cent efficiency. The absorbancy of the acidified CMP and UMP fractions was read at 260 and 280 mμ and their amount determined with the use of the following extinction coefficients ($\epsilon_{260\ m\mu} \times 10^{-3}$): UMP, 9·9 and CMP, 6·2.

The 6-^{14}C-orotic acid (14·6 mc/m-mole) was a product of NAEC Institute, Budapest, Hungary. Uracil-6-acetic acid was a product of Fluka A. G., Switzerland. The synthesis and identification of 5-azaorotic acid were performed as described previously;[8] 2-thioorotic acid was obtained according to ref. 9; orotic acid hydrazide according to ref. 10; 4, 6-dihydropicolinic acid according to ref. 11; the orotaldehyde hydrazone was synthesized by us from orotaldehyde and hydrazine hydrate, the same compound

being described independently by ref. 12. GTP and ATP were obtained from P. L. Laboratories, Milwaukee, Wisconsin: phosphoenolpyruvate, sodium salt from Boehringer A. G., Germany; UMP and CMP were purchased from Calbiochem, Switzerland.

RESULTS

The kinetics of ^{14}C-orotate incorporation into uridine and cytidine nucleotides *in vitro* has been investigated by Genchev.[3] During the time interval used in this study the conversion rate of the ^{14}C-orotate into nucleotides does not change. The labelling of cytidine nucleotides at the end of incubation is about 20-fold lower than that of the uridine nucleotides.

The action of the different compounds tested at a concentration of 0·5 μmoles/ml on the labelling of uridine and cytidine nucleotides *in vitro* is represented in Fig. 2.

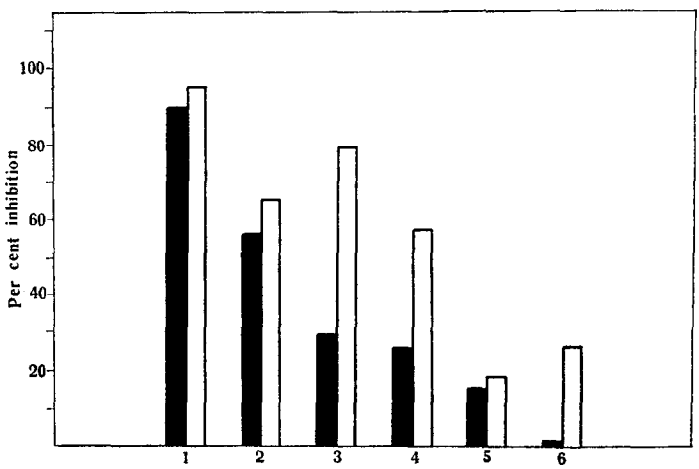

FIG. 2. Inhibition of *in vitro* ^{14}C-orotate incorporation into uridine (black bars) and cytidine (white bars) nucleotides by orotic acid analogues.

The action of the separate compounds at a concentration of 0·5 μmoles/ml was tested in a soluble rat liver enzyme system as described under Methods. The inhibitory action is shown as compared with the control sample with the analogue omitted. (1) Azaorotic acid; (2) orotic acid hydrazide; (3) 2-thioorotic acid; (4) uracil-6-acetic acid; (5) orotaldehyde hydrazone; (6) 4, 6-dihydroxypicolinic acid ("3-deazaorotic acid"). The specific activity expressed as counts/min per μmole nucleotide is of the order of 1.10^6 for UMP and 4.10^4 for CMP.

As can be seen, all the compounds tested inhibit to a varying extent the labelling of UMP and CMP. The strongest inhibitory action on uridine nucleotides synthesis was displayed by 5-azaorotic acid and orotic acid hydrazide, while dihydroxypicolinic acid is devoid of inhibitory activity. In the case of ^{14}C-orotate incorporation into cytidine nucleotides 5-azaorotic acid is again the most efficient, followed by 2-thioorotic acid and orotic acid hydrazide. Dihydroxypicolinic acid has a small, but definite inhibitory action on the synthesis of cytidine nucleotides. It should be noted that with some compounds (orotic acid hydrazide and orotaldehyde hydrazone) the inhibition of UMP and CMP labelling is identical, while with others (2-thioorotic acid and uracil-6-acetic acid) inhibition of CMP labelling is much stronger than that of UMP.

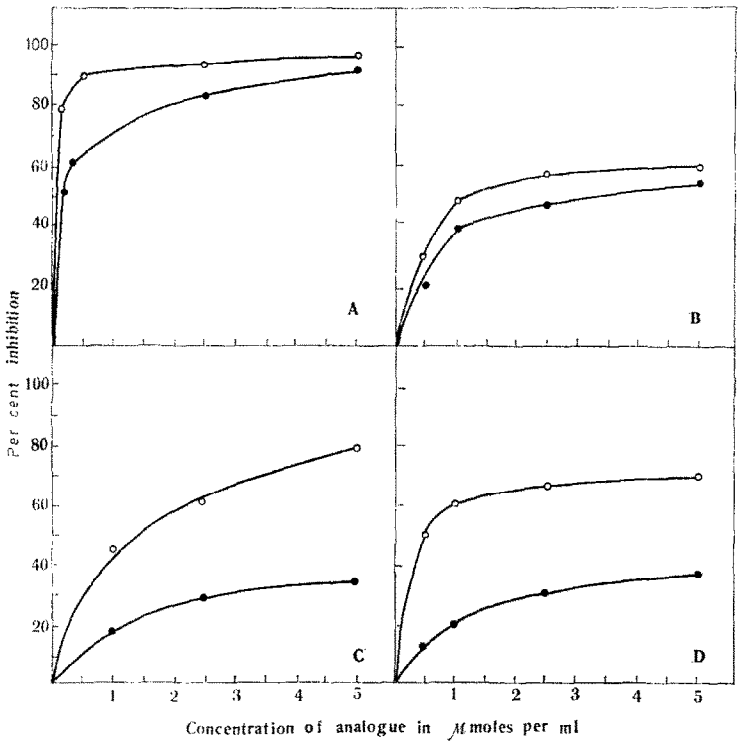

FIG. 3. Concentration dependence of the inhibitory action of orotic acid analogues on the *in vitro* incorporation of ^{14}C-orotic acid into uridine and cytidine nucleotides.

The rat liver soluble enzyme system was as given under Methods. The incorporation into UMP (●—●) and CMP (○—○) is shown as compared to the complete enzyme system taken as control. (A) 5-azaorotic acid. The concentrations of 5-azaorotic acid are 10-fold lower than indicated on the absciss (B) orotic acid hydrazide; (C) 2-thioorotic acid; (D) uracil-6-acetic acid.

In order to study in more detail the observed effect of the pyrimidine analogues concentration dependence of their inhibitory action was investigated. We selected the four most active compounds. The results of these experiments are given in Fig. 3 (A–D). The strongest inhibitory action is displayed by 5-azaorotic acid, which at a concentration of 0·5 μmoles/ml inhibits almost completely the incorporation of ^{14}C-orotate in both uridine and cytidine nucleotides. At lower concentrations of 5-azaorotic acid the inhibition of CMP labelling is more pronounced than that of UMP labelling (see Fig. 3, A). In the case of orotic acid hydrazide the inhibition of UMP and CMP labelling runs parallel at all concentrations tested.

DISCUSSION

Our experimental results show that the compounds tested in this work inhibit the *in vitro* incorporation of ^{14}C-orotate into the pyrimidine nucleotides. These compounds are structural analogues of orotic acid and likely to compete with the latter for the active site of orotidene-5′-phosphate pyrophosphorylase [orotidine-5′ phosphate: pyrophosphate phosphoribosyltransferase (2.4.2.10)]. It is known that compounds like 5-fluoroorotate[1] or 5-azaorotate[2] which can be converted into the

respective nucleotides by a similar soluble enzyme system act as highly efficient antimetabolites. Consequently, all orotic acid analogues, which inhibit the synthesis of pyrimidine nucleotides, are likely to interact enzymatically with 5-phosphoribosyl-1-pyrophosphate. Theoretical considerations concerning the possibilities for such interaction[13] indicate that 5-azaorotic acid, 2-thioorotic acid, orotic acid hydrazide, orotaldehyde hydrazone and uracil-6-acetic acid are likely to act as inhibitors of this enzyme reaction. On the other hand, 4,6-dihydroxypicolinic acid is expected to interact with 5-phosphoribosyl-1-pyrophosphate at a very low rate. Our experimental findings are in complete agreement with these theoretical considerations.

Analysis of ^{14}C-orotate incorporation into uridine and cytidine nucleotides revealed that a characteristic pattern is observed with the separate analogues. It is noteworthy that 5-azaorotic acid, 2-thioorotic acid and uracil-6-acetic acid inhibit more markedly the incorporation into cytidine nucleotides than into uridine nucleotides. This effect may be due to the formation of the nucleotides of the analogues which may further interfere with the synthesis of uridine and cytidine nucleotides. In the case of 5-azaorotic acid it has been shown that it is converted into 5-azaorotidine-5′ phosphate, which in turn inhibits the orotidine-5′ phosphate decarboxylase.[2] Our data show that 5-azaorotate causes a more pronounced inhibition of cytidine nucleotides labelling. This may be correlated with the partial formation of 5-azauridine-5′ phosphate[2] acting as a potential inhibitor of the CTP-synthetase enzyme complex. A similar interpretation may hold true for the observed preferential inhibition of cytidine nucleotides synthesis caused by 2-thioorotic acid and uracil-6-acetic acid.

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