POTENTIATION OF ANTIBACTERIAL EFFECTS OF DIFFERENT PYRIMIDINE ANALOGUES BY N-FORMYLBIURET

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The combination of N-formylbiuret with some pyrimidine analogues of nucleic acid components results in a striking potentiation of their antibacterial effects. The analogues tested were 6-azapyrimidines, 5-azaorotic and 5-fluoroorotic acid, 5-fluorouracil, 5-fluorouridine and 5-fluoro-2'-deoxyuridine. The biological effects of other tested halogenopyrimidines, 5-azapyrimidines, antifolates, uracil and cytosine arabinosides, and of purine analogues were not affected by the simultaneous application with N-formylbiuret.

By studying the biological effects of N-formylbiuret, a degradation product of 5-azauracil[1,2] it was established that this compound possesses antibacterial[2] and antimitotic[3] activity. Upon the simultaneous application of 6-azauridine and N-formylbiuret a pronounced potentiation of antibacterial[2], virostatic[4] and partly cancerostatic[5] effects of this compound was observed. A similar biological effect was found with other N-substituted and N,N'-disubstituted derivatives of biuret[6,7].

In the present communication we took up the possibility of potentiation of the biological effects of other, practically employed analogues of nucleic acid components, by using simultaneous application of N-formylbiuret. Together with this study, we attempted to localize the inhibitory action of this drug in cell metabolism.

EXPERIMENTAL

Reagents. 5- and 6-azapyrimidines were prepared at this Institute as described previously[8]. Similarly, N-formylbiuret[7] was prepared here. Other pyrimidine and purine analogues were from Calbiochem, Los Angeles, and Hoffmann-La Roche, Nutley. L-Dihydroorotic acid, carbamyl phosphate and D,L-ureidosuccinic acid were from Calbiochem. L-Aspartic- [U-^{14}C] acid (26 Ci/mol) was from the Institute for Research, Production and Uses of Radioisotopes, Prague.

Antibacterial activity. Escherichia coli B grown in a mineral synthetic medium[2] with glucose as a carbon source was used in the experiments. Cultivation was carried out under aseptic conditions at 37°C in test-tubes provided with Kapsenberg stoppers. Compounds dissolved directly before the experiment in the medium were added simultaneously, before inoculation of the culture with a drop of *E. coli* suspension.

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Cell-free extract of *E.coli* was prepared from a culture aerated for 8 h at 37°C in 5 l of synthetic medium. Washed cells were suspended under cooling and sonicated (MSE ultrasonic disintegrator, 80 s, 2°C) in 0·05M-Tris-HCl buffer, pH 7·4. Cell debris was centrifuged (10000 *g*, 20 min, 2°C) and the supernatant fraction was divided in small lots and maintained at −20°C. The protein content was determined according to Lowry and coworkers[9].

Carbamyl aspartotranskinase was assayed at 30°C (total volume 1 ml) after adding of 0·2 ml of enzyme extract. The incubation (20 min) was carried out[10] in $2 . 10^{-2}$M-Tris-HCl buffer of pH 8·7 in the presence of 10^{-2}M-Mg^{2+}-ions, $2{\cdot}5 . 10^{-3}$M carbamyl phosphate and 10^{-3}M L-aspartic-[U-^{14}C] acid. The ureidosuccinic-[^{14}C] acid formed was separated chromatographically on Whatman No 1 paper in 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12). Radioactivity was measured after elution on planchets in a gas-flow counter. The enzyme activity is expressed as μmol of ureidosuccinic acid formed.

Dihydroorotase. The incubation was done[11] for 10 min at 37°C in a total volume of 5 ml in $2 . 10^{-2}$M-Tris-HCl of pH 8·0 after adding 0·3 ml of enzyme extract in the presence of $5 . 10^{-3}$M EDTA and $1{\cdot}5 . 10^{-3}$M L-dihydroorotic acid. The enzyme activity was determined from the decrease of absorbance at 240 nm and is expressed as μmol of ureidosuccinic acid formed. It is a disadvantage of the method that the ureidosuccinic acid formed undergoes simultaneous recyclization to 5-carboxymethylhydantoin[12].

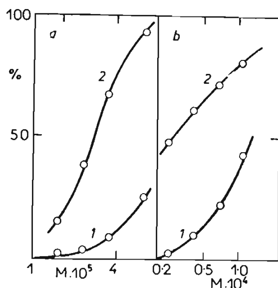

Fig. 1

Potentiation of Antibacterial Activity of 6-Azauracil (*a*) and 6-Azacytosine (*b*) by N-Formylbiuret

The compounds were added to the medium before inoculation either alone (*1*) or in combination with $2 . 10^{-5}$M N-formylbiuret (*2*) which does not affect bacterial growth at this concentration when present alone. Static cultivation of *E. coli* B for 16 h at 37°C. %, Inhibition of bacterial growth; the culture growth without inhibitor = 100%. *M*, Concentration of added analogue.

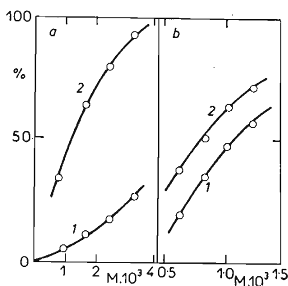

Fig. 2

Different Effects of N-Formylbiuret on the Antibacterial Acitivity of 6-Azauridine (*a*) and 5-Azauridine (*b*)

The compounds were added to the medium before inoculation either alone (*1*) or in combination with $2 . 10^{-5}$M N-formylbiuret (*2*). For details see Fig. 1.

RESULTS AND DISCUSSION

It was found that, as in case of 6-azauracil and 6-azauridine[2], simultaneous application of N-formylbiuret results in an enhanced antibacterial activity of 6-azacytosine and 6-azacytidine (Figs 1–3). The antibacterial effects of 6-azathymine and 6-azathymidine were not affected by N-formylbiuret. The mechanism of action of the thymine analogues differs[13] from the inhibitory mechanism of potentiated 6-azapyrimidines which inhibit practically selectively the *de novo* synthesis of pyrimidines[14]. In contrast with 6-azapyrimidines, the antibacterial effect of the corresponding 5-azaanalogues[15] is not affected by the presence of N-formylbiuret (Figs 1–3). It is known that these compounds, similarly to 6-azapyrimidines, interfere with the *de novo* synthesis of pyrimidines[16]. However, due to spontaneous and enzymic transformations and incorporation into different types of nucleic acids their effect appears to be polyvalent[17]. An exception among 5-azapyrimidines is represented by 5-azaorotic acid[18,19] (oxonate), the inhibitoty effect of which is increased by the combination with N-formylbiuret (Fig. 4). A similar behaviour was found with 5-fluoroorotic acid even if the two compounds differ in the mechanism of their inhibitory effect[20,21]. A common feature of the two compounds undergoing the same metabolic transformation is an interference with the pyrimidine pathway[18–20]. The last group of compounds, the antibacterial efficiency of which is distinctly increased by combination with N-formylbiuret are the 5-fluoroanalogues of uracil[22,23] (Fig. 5). The antibacterial effect of other 5-halogenosubstituted analogues of uracil was not potentiated by combination with N-formylbiuret. Also the antibacterial

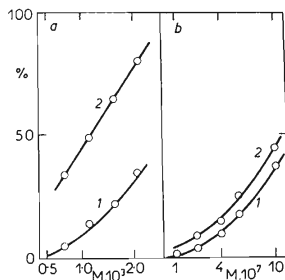

FIG. 3

Effect of N-Formylbiuret on the Antibacterial Activity of 6-Azacytidine (*a*) and 5-Azacytidine (*b*)

For details see Fig. 1.

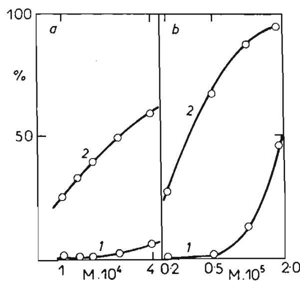

FIG. 4

Increase of Antibacterial Activity of 5-Substituted Analogues of Orotic Acid on Combination with N-Formylbiuret

a 5 Azaorotate, *b* 5-fluoroorotate. For details see Fig. 1.

effect of tested antifolates, uracil and cytosine arabinosides and of a number of purine analogues of nucleic acid components (6-mercaptopurine, 8-azaguanine, 8-azaadenine and adenine arabinoside) was not affected by simultaneous application with N-formylbiuret.

When evaluating the antibacterial activity of N-formylbiuret and its ability to potentiate the effects of other compounds one must take into consideration its instability. Loss of the formyl group results in the formation of buiret[1,2] while cyclization leads to 5-azauracil[2] which, however, cannot potentiate the biological activity of the compounds studied. On the other hand, 5-azauracil[2], 5-azacytidine[15] but also 5-azaorotic acid[24] may give rise under suitable conditions to N-formylbiuret. This fact complicates the problem why in the presence of N-formylbiuret the antibacterial effect of 6-azapyrimidines but not that of 5-azapyrimidines with the exception of 5-azaorotic acid is potentiated.

The biological activity of N-formylbiuret may be reversed by the simultaneous application of natural pyrimidine components of nucleic acids[2]. It would thus appear that the inhibitory action of N-formylbiuret is localized in the pyrimidine pathway. However, the investigation of enzymes of the pyrimidine pathway in the presence of N-formylbiuret (Table I) did not provide evidence for this inhibition. Orotate phosphoribosyltransferase and orotidylic acid decarboxylase were not effected while carbamyl aspartotranskinase and dihydroorotase were affected to a minor degree (12·4 and 21·7% inhibition, respectively) at a concentration of 10^{-3}M N-formylbiuret. The finding may be extended by the observation that also a combination of N-formylbiuret with 6-azauridine when the biological effect is potentiated does not substantially affect the activity of enzymes of the pyrimidine pathway *in vitro*. On the basis of these findings we assume that the previously observed inhibition of *E. coli* dihydroorotase[1] cannot be considered as the only site of inhibitory action of N-formylbiuret. The fact that the biological activity of N-formylbiuret may be removed by adding pyrimidine precursors without any substantial effect on their synthesis and utilization, indicates the possibility of N-formylbiuret interfering in other areas of cell metabolism.

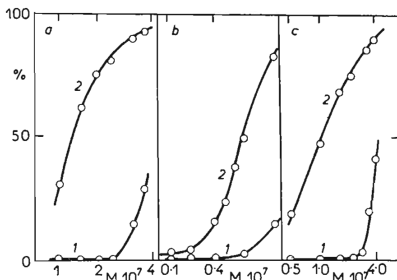

FIG. 5

Potentiation of Antibacterial Activity of 5-Fluoropyrimidines in Combination with N-Formylbiuret

a 5-Fluorouracil, *b* 5-fluorouridine, *c* 5-FUdR. For details see Fig. 1.

TABLE I

Effect of N-Formylbiuret on the Enzymes of *de novo* Pyrimidine Synthesis

Calculated on the basis of 3 independent experiments using a cell-free *E. coli* extract as a source of enzyme activity.

Enzyme	Activity, μmol/ml		Inhibition %
	Control	10^{-3}M N-formylbiuret	
Carbamyl aspartotranskinase	0·73 ± 0·12	0·64 ± 0·08	12·4
Dihydroorotase	0·23 ± 0·03	0·18 ± 0·03	21·7
Orotate phosphoribosyltransferase[a]	0·049 ± 0·008	0·051 ± 0·006	0
Orotidylic acid decarboxylase[a]	0·038 ± 0·005	0·038 ± 0·007	0

[a] Activity of enzymes was assayed as described earlier[16,19].

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