THE MOLECULAR MECHANISM OF ANTIBACTERIAL ACTIVITY OF 5-SUBSTITUTED HALOGEN DERIVATIVES OF 2,3-DIHYDRO-1,3-6*H*-OXAZINE-2,6-DIONE ("3-OXAURACIL")

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5-Substituted halogen derivatives of "3-oxauracil" (5-chloro, 5-bromo- and 5-iodo-3-oxauracil) act as potent inhibitors of growth of *Escherichia coli*. Their inhibitory effect cannot be reversed by preformed pyrimidines and none of the derivatives studied displays mutagenic activity toward *E. coli*. In the growth medium 5-substituted halogen derivatives of 3-oxauracil are rapidly decomposed, giving rise to halogenoacetaldehydes which interact with cell proteins and are thus responsible for the inhibitory effect of the original compounds. Growth inhibition caused by the 5-substituted halogen derivatives of 3-oxauracil (like that by the corresponding halogenoacetaldehydes) can thus be relieved by glutathione.

Research on the biological activities of "3-oxauracil" and its nucleosides dates back only a few years[1-5]. It was then stimulated by the discovery of a new antibiotic, oxazinomycin[6], the structure of which was determined as 5-β-D-ribofuranosyl-3,4-dihydro-1,3-2*H*-oxazine-2,4-dione[7,8]. 3-Oxauracil was found to be a potent inhibitor of growth of various cell types; its antimicrobial activity attains the level of antibiotics in some cases.

Heidelberger and coworkers[4] proceeded from the assumption that 3-oxauracil can react by virtue of its anhydride function with electrophilic centres and bind covalently to the active sites of some enzymes participating in the metabolism of pyrimidines. However, a definite proof that 3-oxauracil is one of the active-site directed irreversible inhibitors of one of the enzymes involved, is still lacking. The present results actually indicate that the inhibition of bacterial growth brought about by 3-oxauracil is of a strictly competitive character[1,2].

The above-quoted authors[4] compared the growth-inhibiting action of 3-oxauracil and of its ribonucleoside and found the activities of the two compounds to be the same with respect to a culture of L 5178Y cells[3]. The oxazine analogue of thymine, 5-methyl-3-oxauracil, inhibited the growth of some microbial and tumour cells with a much lower intensity than 3-oxauracil[5]. On the other hand, the 2′-deoxyribonucleoside of 3-oxauracil exhibited a thousand-fold greater inhibitory activity toward *S. faecium* than did 3-oxauracil; this inhibition was partly reversed by uridine, 2′-deoxyuridine, cytidine, 2′-deoxycytidine and thymidine[5]. Likewise, the inhibitory effect of 3-oxauracil on the growth of *E. coli* B was completely removed by uridine, cytidine and partly by uracil; other natural pyrimidine and purine precursors did not relieve the inhibition of growth caused by 3-oxauracil[1].

Further experiments were influenced by the attempt to localize more closely the growth-inhibiting effect of 3-oxauracil. The inhibitor was found to affect synthesis of DNA and

RNA to the same degree in growing cells of *E. coli* B. A secondary sign of inhibition of RNA biosynthesis is seen in the decreased rate of protein synthesis. At the same time, in a subcellular system of *E. coli* B 3-oxauracil does not inhibit any of the enzymes responsible for the synthesis of uridine 5'-phosphate. In agreement with these findings, the growth-inhibiting effect of 3-oxauracil was found to be unaffected by any of the precursors of *de novo* synthesis of uridine 5'-phosphate from aspartic acid[2]. The molecular mechanism of the effect of 3-oxauracil which brings about a rapid inhibition of RNA and DNA biosynthesis in growing cells of *E. coli* B thus remains unknown.

Interesting biological activities of some halogen derivatives of natural pyrimidine bases (5-fluorouracil, 5-bromouracil, 5-iodouracil) led us to check the growth-inhibiting properties of the halogen derivatives of 3-oxauracil (5-chloro, 5-bromo and 5-iodo--2,3-dihydro-1,3-6*H*-oxazine-2,6-dione). The series of halogen derivatives tested was extended by 5-methyl-3-oxauracil (5-methyl-2,3-dihydro-1,3-6*H*-oxazine-2,6--dione), the biological effects of which have been mentioned by American authors[5]. Preliminary experiments indicated that the growth-inhibiting effects of 5-substituted halogen derivatives of 3-oxauracil are probably based on other mechanisms than the effect of unsubstituted 3-oxauracil. In view of the hydrolysis of these derivatives it was decided here to follow the inhibitory effects of the original compounds as well as of their degradation products.

EXPERIMENTAL

Material

The methods of preparation and the principal characteristics of 3-oxauracil and all the 5-substituted derivatives of 3-oxauracil were reported previously[9,10]. The other pyrimidine precursors were obtained from Calbiochem (USA). Chemicals used for preparing the buffers and cultivation media were of analytical purity.

Estimation of the Growth-Inhibiting Effects of 5-Substituted Derivatives of 3-Oxauracil and of Their Degradation Products

The growth-inhibiting effect of 5-substituted derivatives of 3-oxauracil on *E. coli* was examined in a minimal synthetic medium with inorganic salts and glucose[1]. The medium with the appropriate amount of inhibitor was inoculated with a 16 h culture of bacteria grown in the same medium. Cell growth was assayed after 16 h of stationary cultivation at 35°C from absorbance at 575 nm. The prototrophic strain *E. coli* B and the auxotrophic strain *E. coli* WP 14 Pro$^-$ were used for testing the growth-inhibiting effects of 3-oxauracil derivatives, the cultivation medium for the latter being enriched with 10^{-3}M L-proline.

The growth-inhibiting effects of the hydrolytic products of 5-substituted halogen derivatives of 3-oxauracil was examined in a similar way. Before addition to the growth medium solutions of the halogen derivatives of 3-oxauracil were hydrolyzed by 0·01M-NaOH for 2 h at 25°C. The completion of hydrolysis of the 5-substituted halogen derivatives of 3-oxauracil to the corresponding halogenoacetaldehydes was checked spectrophotometrically[10]. The reference compound used here was an authentic sample of bromoacetaldehyde.

The possibility of the preventing the growth-inhibiting effect of the halogenoacetaldehydes by glutathione was examined under the above conditions. The cultivation medium contained here glutathione in a twice greater molar concentration than that of the inhibitor used.

The potential metabolic antagonism of 5-substituted derivatives of 3-oxauracil with the pyrimidine precursors of nucleic acids was followed in the *E. coli* B strain on a minimal synthetic medium[1]. The growth medium containing the inhibitory amount of the 3-oxauracil derivative and one of the pyrimidine precursors of nucleic acids in a molar ratio of 1 : 1 or 1 : 10, was combined with a 1% inoculum of a 16 h culture and samples were incubated for 16 h in a batch arrangement at 35°C. Growth of bacteria was assayed from absorbance at 575 nm.

Estimation of the Revertant Frequency of *E. coli* WP 14 Pro$^-$ Growing in the Presence of 5-Substituted Derivatives of 3-Oxauracil

The auxotrophic strain *E. coli* WP 14 Pro$^-$ was grown in a batch culture for 16 h at 35°C in a minimal medium[1] enriched with 10^{-3}M L-proline and containing the tested derivative of 3-oxauracil or one of the reference compounds. The concentrations of these compounds were chosen to depress bacterial growth by 20—60%. Culture samples were then centrigufed, bacterial sediments washed with physiological saline and restored to the original volume. Cell suspensions were diluted serially ten-fold. Samples of suitably diluted suspensions (0·1 ml) were placed on Petri dishes with meat-peptone agar and with minimal medium (synthetic medium solidified with 1·5% agar). After 16 h at 35°C the total cell count was determined in 1 ml original suspension; after 40 h of incubation cell colonies were counted on plates with the minimal medium and in this way the number of revertants in 1 ml original suspension was estimated. The mutagenic activity of the compounds tested was expressed by the number of revertants per 10^{12} viable cells.

Study of Hydrolysis of the 5-Substituted Derivatives of 3-Oxauracil

The rate of hydrolysis of 3-oxauracil derivatives under the conditions of bacteriological tests was followed by a method described earlier[2]. 1 mM solutions of 5-substituted derivatives of 3-oxauracil in the minimal medium with glucose[1] were incubated for 16 h at 35°C. At 2 h intervals 1 ml samples of the reaction mixture were pipetted into 9 ml 0·2M-HCl and absorbance was estimated at the wavelengths of the absorption maxima of the compounds tested.

RESULTS AND DISCUSSION

Growth-Inhibiting Effects of 5-Substituted Derivatives of 3-Oxauracil

The growth-inhibiting effect of 5-substituted derivatives of 3-oxauracil was examined in *E. coli* B and *E. coli* WP 14 Pro$^-$ and both strains were found to respond to the compounds by the same degree of inhibition. Fig. 1 shows the most active inhibitors of growth to be 5-iodo-3-oxauracil and 5-bromo-3-oxauracil. Both compounds are much active antibacterially than unsubstituted 3-oxauracil. The chloro derivative showed a much weaker activity in these tests; the growth-inhibiting effect of 5-methyl-3-oxauracil was lower by two orders of magnitude than that of the most potent halogen derivatives. The different slope of the inhibition curve of unsubstituted

3-oxauracil as compared with the slopes of the inhibition curves of its 5-substituted derivatives (Fig. 1) permits us to infer a different molecular mechanism of inhibition of microbial growth.

Antagonism of 5-Substituted Derivatives of 3-Oxauracil and the Natural Pyrimidine Bases

The investigation of antagonisms between the 5-substituted derivatives of 3-oxauracil and the natural pyrimidine bases or their nucleosides supported earlier assumptions. The growth-inhibiting activity of the minimum inhibitory amount of none of the 3-oxauracil derivatives was diminished by thymine or uracil or by their deoxyribo- or ribonucleosides, even if present in a 10-fold excess over the inhibitors. We are thus apparently dealing with a difference in the mechanism of antibacterial action between the halogen derivatives of 3-oxauracil and the 3-oxauracil itself, the growth--inhibiting effect of which can be fully reversed by preformed pyrimidines.

Study of Potential Mutagenic Effects of 5-Substituted Derivatives of 3-Oxauracil

If 5-substituted derivatives of 3-oxauracil were mutagenic nucleic acid might be target compounds. Hence the analogues were tested in connection with the reversion frequency of the auxotrophic strain *E. coli* WP 14 Pro$^-$ into its prototroph. The reference mutagens used were the highly active 5-azacytidine[11] and the inactive 6-azauracil[6] which is known to make the affected codon triplets nonfunctional but not to cause errors in the translation process[11,12]. From a comparison of the number of revertants it appeared that none of the 5-substituted derivatives of 3-oxauracil exhibited a mutagenic effect while 5-azacytidine was a potent mutagen under the same conditions (Table I). This fact leads to the assumption that in the auxotrophic strain tested these uracil analogues are not incorporated into nucleic acids.

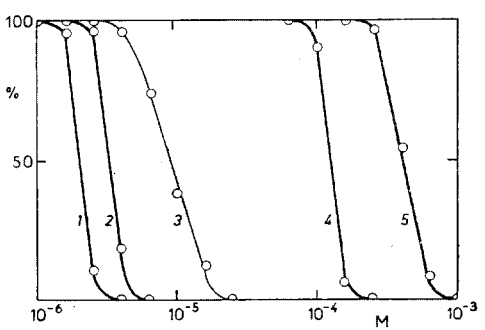

FIG. 1

Inhibition Curves of 3-Oxauracil and Its 5-Substituted Derivatives

M molar concentration of inhibitor, % growth of bacteria referred to the control. *1* 5-Iodo-3-oxauracil, *2* 5-bromo-3-oxauracil, *3* 3-oxauracil, *4* 5-chloro-3-oxauracil, *5* 5--methyl-3-oxauracil. Experimental conditions are described in the text.

Hydrolysis of 5-Substituted Derivatives of 3-Oxauracil and the Growth-Inhibiting Activity of Their Degradation Products

In view of the relatively rapid degradation of the 5-substituted derivatives of 3-oxauracil in an alkaline medium[10] we considered it useful to determine the rate of hydrolysis of 3-oxauracil derivatives under the conditions of bacteriological tests. In agreement with earlier observations on the dependence of the hydrolysis rate on pH, the half-times of degradation of the various 5-substituted derivatives were greater than those measured in an alkaline medium. In comparison with 3-oxauracil and 5-methyl--3-oxauracil, the 5-substituted halogen derivatives were more disposed to hydrolytic degradation[10] (Table II). The character of the UV spectra of the reaction mixtures permits us to conclude that the hydrolysis of the 5-substituted derivatives of 3-oxauracil in a cultivation medium of pH 7·4 proceeds by the same mechanism as the previously investigated hydrolysis in an alkaline medium and that the final products are the corresponding substituted acetaldehydes[10].

The observed differences in the inhibitory action of 3-oxauracil and its 5-substituted derivatives, as well as the more rapid hydrolytic degradation of 5-substituted halogen derivatives of 3-oxauracil led us to checking the possible growth-inhibiting properties of the degradation products of these compounds. Earlier experiments suggested that 3-oxauracil and its 5-substituted derivatives are decomposed *via* intermediates to formylacetic acid and its derivatives, or even to derivatives of acetaldehydes[10]. Degradation of 3-oxauracil or 5-methyl-3-oxauracil thus gives rise to the (at the given concentration nontoxic) acetaldehyde or propionaldehyde while in the case of 5-substituted halogen derivatives of 3-oxauracil halogenoacetaldehydes are

TABLE I

Effect of Pyrimidine Analogues on Revertant Frequency of *Escherichia coli* WP 14 Pro$^-$

Compound	Number of revertants per 10^{12} cells
0	25
3-Oxauracil	35
5-Methyl-3-oxauracil	70
5-Chloro-3-oxauracil	70
5-Bromo-3-oxauracil	40
5-Iodo-3-oxauracil	50
5-Azacytidine	1 230
6-Azauracil	30

TABLE II

Half-Times of Degradation of 5-Substituted Derivatives of 3-Oxauracil under Conditions of Bacteriological Tests

Compound	Degradation half-time
3-Oxauracil	10 h
5-Methyl-3-oxauracil	8 h
5-Chloro-3-oxauracil	3 h 40 min
5-Bromo-3-oxauracil	4 h
5-Iodo-3-oxauracil	5 h

formed, the growth inhibiting effects of which were examined. The 5-halogen derivatives of 3-oxauracil subjected to alkaline hydrolysis were found to inhibit the growth of *E. coli* B to the same degree as the original substances. It is thus probable that the proper inhibitory agent are the hydrolytic products of the 5-halogen derivatives of 3-oxauracil, *viz* the halogenoacetaldehydes. This possibility was supported by the experiment carried out with an authentic sample of bromoacetaldehyde which inhibited the growth of *E. coli* B with the same intensity as a solution of 5-bromo--3-oxauracil after alkaline hydrolysis. The actual cause of inhibitory action of halogenoacetaldehydes is a nonspecific interaction of these potent alkylating agents with the SH-groups of the active sites of cell proteins. This view was supported by the experiments with glutathione which relieved completely the inhibition of bactetial growth caused both by the halogenoacetaldehydes and by the 5-halogeno derivatives of 3-oxauracil.

On the basis of these results the view is advanced that the 5-substituted halogeno derivatives of 3-oxauracil do not act in the systems followed as antimetabolites of the natural pyrimidine bases. Their growth-inhibiting activity could never be removed by preformed pyrimidines and none of the 5-halogeno derivatives of 3-oxauracil exhibited mutagenic activity that would suggest its incorporation into deoxyribonucleic acid. The 5-substituted halogen derivatives of 3-oxauracil thus exhibit a fundamentally different mechanism of action than unsubstituted 3-oxauracil.

The results of the experiments reported thus permit to make the conclusion that the growth-inhibiting activity of 5-substituted halogen derivatives of 3-oxauracil is due to their degradation products, in particular halogenoacetaldehydes which, as powerful alkylating agents, offer wide possibilities of nonspecifically interfering with the active sites of enzymes in different metabolic pathways.

REFERENCES

1. Škoda J., Flegelová Z., Farkaš J.: Biochem. Biophys. Res. Commun. *50*, 80 (1973).
2. Škoda J., Votruba I., Farkaš J.: This Journal *39*, 1500 (1974).
3. Chwang T. L., Heidelberger C.: Tetrahedron Lett. *1974*, 95.
4. Heidelberger C.: Cancer Res. *30*, 1549 (1970).
5. Bobek M., Bloch A., Kuhar S.: Tetrahedron Lett. *1973*, 3493.
6. Haneishi T., Okazaki T., Hata T., Tamure C., Nomura M., Naito A., Seki I., Arai M.: J. Antibiot. *24*, 797 (1971).
7. Kusakabe Y., Nagatsu J., Shibuya M., Kawaguchi O., Hirose O., Shirato S.: J. Antibiot. *25*, 44 (1972).
8. Sasaki K., Kusakabe Y., Esumi S.: J. Antibiot. *25*, 151 (1972).
9. Farkaš J., Škoda J.: Czech. Appl. PV 214—73 (1973).
10. Farkaš J., Fliegerová O., Škoda J.: This Journal *41*, 2059 (1976).
11. Fučík V., Zadražil S., Šormová Z., Šorm F.: This Journal *30*, 2883 (1965).
12. Lisý V., Škoda J., Rychlík I., Smrt J., Holý A., Šorm F.: This Journal *33*, 4111 (1968).

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