STUDY OF THE MECHANISM OF THE GROWTH-INHIBITING EFFECT OF 3-OXAURACIL IN *Escherichia coli*

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Hydrolysis of 3-oxauracil at 37°C in an aqueous medium was shown to give rise to formylacetic acid as a sole product of degradation. The rate of hydrolysis of 3-oxauracil increases with rising pH value. 3-Oxauracil in *Escherichia coli* inhibits primarily the biosynthesis of DNA and RNA; the observed inhibition of protein synthesis is probably of secondary character. These inhibitory effects may be partly reversed by uridine. In a subcellular system of *E. coli*, 3-oxauracil does not inhibit the metabolic conversions of dihydroorotic and orotic acids; the growth-inhibiting effect of 3-oxauracil cannot be relieved by aspartate, ureidosuccinate, dihydroorotate and orotate. Only preformed pyrimidines (uracil, uridine) antagonize completely the inhibition of bacterial growth due to 3-oxauracil.

In a previous communication[1] we described the growth-inhibiting properties of 3-oxauracil. The compound was first prepared in 1927 (ref.[2]) but its biological activity has remained unknown. It was found here to inhibit the growth of *E. coli* B at concentrations comparable with the effective concentrations of antibiotics. The inhibitory effect can be completely relieved by uracil or uridine[1].

The present communication describes studies designed to localize the inhibitory effect of 3-oxauracil on the metabolic processes of *E. coli*. These detailed studies were stimulated by experiments where 3-oxauracil was found to be a potent inhibitor of growth of a number of Gram-positive as well as Gram-negative bacteria.

EXPERIMENTAL

Materials

3-Oxauracil was prepared by a new method[3] based on the oxidation of maleic monoamide with lead tetraacetate in pyridine. M.p. 156–158°C (decomp.); ref.[2,4] give 158°C and 158–159°C, respectively. IR spectrum (in KBr): 1 634 cm^{-1} (C=C), 1 715 cm^{-1} (C=O), 1 785 cm^{-1} (C=O). The IR spectrum is identical with that of the compounds obtained in the reaction[4] of trimethylsilyl azide with maleic anhydride and subsequent hydrolysis. NMR spectrum (100 MHz) in hexadeuteriodimethyl sulfoxide (p.p.m.): δ 5·48 (d, 1 H, $J_{5,4}$ 7·4 Hz, H_5), 7·46 (d, 1 H, $J_{4,5}$ 7·4 Hz, H_4), 11·40 (broad s., 1 H, $J_{3,4}$ 0 Hz, H_3). UV spectrum in 0·1M-HCl (pH 1·20): λ_{max} 264 nm (log ε 3·949); in 0·1M borate buffer (pH 11·2): λ_{max} 296 nm (log ε 4·046). The UV spectra at various

pH values are shown in Fig. 1. The pK value is 7·78 ± 0·02 (estimated spectrophotometrically[5] at 25°C using a series of 0·067M phosphate buffers, the pH values being increased in 0·1 unit steps approximately).

All other chemicals were from Calbiochem (U.S.A.). Orotic acid-[6-^{14}C] was obtained from New England Nuclear Corporation (U.S.A.), D,L-dihydroorotic acid-[6-^{14}C] was prepared from this radioactive orotic acid according to Cooper and coworkers[6]. The specific radioactivity of these two substrates was 4·5 mCi/mmol. L-Leucine-U-[^{14}C] was made at the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia), its specific activity being 79 mCi/mmol. Orthophosphate[^{32}P] was a carrier-free preparation from Isocomerz (German Democratic Republic).

Kinetics of 3-Oxauracil Hydrolysis

Dependence of hydrolysis rate on pH. 3-Oxauracil (59·1 mg, 0·523 mmol) was dissolved in a 50-ml volumetric flask in water whereafter 5 ml amounts of the solution were transferred to six 50-ml flasks. The volumes were made up to 50 ml with: *1*) 0·1M-HCl, *2*) 0·1M citrate buffer of pH 5·24, *3*) 0·067M phosphate buffer of pH 7·15, *4*) 0·067M phosphate buffer of pH 7·56, *5*) 0·1M borate buffer of pH 10·38, *6*) 0·1M borate buffer of pH 11·36. The flasks were placed in a water bath kept at 37·1 ± 0·1°C with a Wobser U8 thermostat. At one- and two-hour intervals, 5 ml aliquots were removed and made up to 50 ml with 0·2M-HCl and the absorbance at 264 nm estimated using 0·2M-HCl as a blank.

Hydrolysis in a neutral medium. About 20 mg 3-oxauracil was placed in each of 4 volumetric flasks and the volume made up to 100 ml with the following solutions: *1*) 0·01M phosphate buffer of pH 7·58, *2*) 0·1M phosphate buffer of pH 7·36; *3*) a synthetic cultivation medium[1] without glucose, of pH 6·98, *4*) 0·02M Tris-HCl buffer of pH 7·61. The flasks were placed in a water bath at 37·1 ± 0·1°C. Five-ml samples were withdrawn in 2-hour intervals and the volume made up in (*1*) (*2*) (*3*) to 100 ml with 0·2M-HCl, in (*4*) to about 35 ml whereafter 3 ml Dowex 50(H^+)

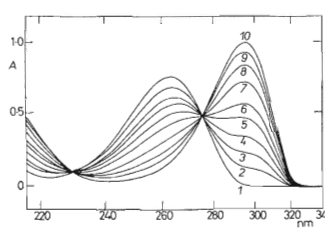

FIG. 1

UV Spectra at Different pH Values

8·67 . 10^{-5}M solution of 3-oxauracil in the following buffers: *1* glycine-HCl at pH 1·18; *2* 0·067M phosphate at pH 6·93; *3* 0·067M phosphate at pH 7·28; *4* 0·067M phosphate at pH 7·48; *5* 0·067M phosphate at pH 7·71; *6* 0·067M phosphate at pH 7·88; *7* 0·1M borate at pH 8·19; *8* 0·1M borate at pH 8·46; *9* 0·1M borate at pH 8·87; *10* 0·1M borate at pH 9·85.

was added. After 5 min of standing and occasional stirring, the resin was filtered, washed with 20 ml water and the filtrate made up to 100 ml with 0·2M-HCl. The spectrophotometric estimation of 3-oxauracil was performed as described above. To estimate formylacetic acid, 5 ml of the reaction mixture was pipetted off and the volume made up with 0·1M-NaOH to 50 ml; absorbance at 258 nm was measured against 0·1M-NaOH as a blank.

Paper chromatography of reaction mixtures after a 16-hour hydrolysis: Samples of the reaction mixture (5 ml) were placed on a half sheet of Whatman 3MM paper and developed in 1-butanol–acetic acid–water (20 : 7 : 3). The compounds were detected either under UV light or by spraying with 0·1% $KMnO_4$.

Microorganism

Inhibition of growth and antagonism of 3-oxauracil with pyrimidine precursors of nucleic acids were assayed in a medium with inorganic salts and glucose[1]. The inhibitor as well as the precursors were present in the medium before adding 1% inoculum of *E. coli* B, grown in the above medium[1]. Growth was estimated after 15 h of cultivation at 37°C by measuring absorbance at 575 nm.

Preparation of Cell-Free Extracts

E. coli B was grown in 4000 ml synthetic medium of the above composition at 37°C. The bacteria were harvested by centrifugation at 4°C, either at the onset of the logarithmic phase of growth ("young cells"), or after attaining stationary growth phase ("old cells"). The centrifuged bacteria were washed with 0·2M Tris-HCl buffer of pH 7·4, suspended in a small volume of the buffer and sonicated for 105 s (MSE ultrasonic disintegrator, Great Britain). The sonicated suspensions were freed of the cell debris by centrifugation at 30000 *g* in the cold and the supernatants were divided into small portions which were stored at −70°C. The total protein content determined according to Lowry and coworkers[7] was 32 mg/ml in the "young-cell" supernatant, 75 mg/ml in the "old-cell" one.

Incorporation of Orthophosphate-[^{32}P] into Nucleic Acids and of Leucine-[^{14}C] into Proteins of Bacteria

The incorporation of orthophosphate-[^{32}P] into bacterial nucleic acids was followed in a medium with a low phosphorus content[8], using 6 μCi orthophosphate-[^{32}P] (204 μg) per ml. Aliquot parts of the culture were pipetted at suitable time intervals into a solution of KOH (final concentration 0·33M) and hydrolyzed at 37°C for 16 h. At the same time, the same aliquot part was pipetted into ice-cold trichloroacetic acid (final concentration 5%). Using filtration through membrane filters (Synpor 6, Synthesia, Czechoslovakia) the radioactivity of the trichloroacetic acid-insoluble fraction was determined (RNA + DNA) as well that of the fraction resisting hydrolysis (DNA).

Incorporation of leucine-[^{14}C] was followed in the synthetic medium mentioned above[1]; 1 ml of the medium contained 0·2 μCi leucine-[^{14}C] (20 μg). The degree of incorporation was determined after filtration on membrane filters in the fraction insoluble in hot 5% trichloroacetic acid. Radioactivity trapped on the membrane filter was counted by liquid scintillation on a Packard 3375 spectrometer (U.S.A.).

Metabolism of Dihydroorotic and Orotic Acids in the Presence of Cell-Free Extracts

The metabolic conversions of dihydroorotic acid were examined in the following medium: $2 . 10^{-2}$M Tris-HCl buffer of pH 7·4, $2 . 10^{-4}$M-$MgSO_4$, $2 . 10^{-4}$M-NAD, $5 . 10^{-4}$M D,L-dihydro-

orotic acid-[6-^{14}C], $5 \cdot 10^{-4}$M 3-oxauracil and 2·5 mg proteins of the cell-free extract of cells harvested at the stationary growth phase. The total volume of the incubation mixture was 0·8 ml.

The metabolic conversions of orotic acid were followed in the following medium: $2 \cdot 10^{-2}$M Tris-HCl buffer of pH 7·4, 10^{-3}M-$MgSO_4$, $2 \cdot 10^{-3}$M 5-phosphoribosyl-1-pyrophosphate (dimagnesium salt), $5 \cdot 10^{-4}$M (or $5 \cdot 10^{-3}$M) 3-oxauracil and 3·5 mg protein of the cell-free extract (*a*) from "young" as well as (*b*) "old" cells. The mixture was preincubated for 10 min to allow for any phosphoribosylation of 3-oxauracil whereafter orotic acid-[6-^{14}C] was added to a final concentration of $5 \cdot 10^{-4}$M. The total volume of the incubation mixture was 0·6 ml.

These mixtures were incubated in a water bath at 37°C for 15, 30 and 60 min. After terminated incubation, 0·1 ml samples of the mixture were applied to the paper sheet on a five-cm start line, drying in a stream of air. The chromatograms were developed three times in a mixture of isopropyl alcohol–ammonia–water (7 : 1 : 2) until the solvent front reached the paper end. Reference samples chromatographed at the same time and detected under UV light indicated the position of the compounds examined in the analyzed samples. Paper strips were then cut out from the chromatograms and the distribution of radioactivity was scanned by a Frieseke-Hoepfner automatic recorder.

Spectra

The IR spectrum was determined in a UR-10 (Zeiss, Jena) spectrophotometer, the NMR spectrum in a Varian HA-100 spectrometer. The UV spectra were measured in a Specord (Zeiss, Jena) spectrophotometer. pH was estimated in a Beckman model 1019 pH-meter.

RESULTS AND DISCUSSION

Stability of 3-Oxauracil

When recording the UV spectra of 3-oxauracil in 0·05M-NaOH it was observed (Fig. 2) that the absorbance at 296 nm decreases steeply and a new absorption maxi-

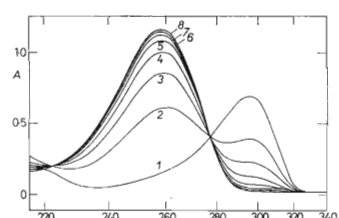

FIG. 2

3-Oxauracil Hydrolysis in an Alkaline Medium Followed by UV Spectra

$6 \cdot 5 \cdot 10^{-5}$M solution of 3-oxauracil in 0·05M-NaOH at 25°C. *1* 0 min, *2*—*8* measured at 10 min intervals.

mum at 258 nm was observed which disappeared on acidifying the solution to pH 4. On the basis of this spectroscopic behaviour, the new maximum might be assigned to the enol-form of formylacetic acid,* an authentic sample of which was prepared by an alkaline hydrolysis of a polymeric formyl ketone[9].

The identity of the hydrolytic product with formylacetic acid was further demonstrated by electrophoresis in 0·067M phosphate buffer of pH 6·9. The electrophoresis was carried out on Whatman 3MM paper at 15 V/cm, for 2 h, using detection with 0·1% $KMnO_4$. The electrophoretic mobility of the authentic sample of formylacetic acid was 16·2 cm, that of the mixture after alkaline hydrolysis of 3-oxauracil also 16·2 cm (3-oxauracil remains at the start; picric acid as a visible reference compound migrated 9·5 cm).

The effect of pH on the rate of 3-oxauracil hydrolysis was followed at 37°C at six values of pH. The curves (Fig. 3) show the 3-oxauracil to be relatively stable in an acid solution; with increasing pH the rate of its hydrolysis is seen to rise steeply.

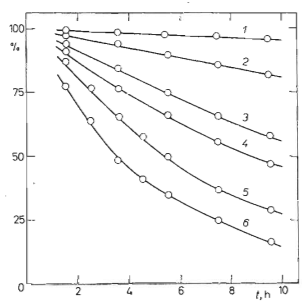

FIG. 3

3-Oxauracil Hydrolysis in Dependence on pH

$1{\cdot}04 \cdot 10^{-3}$M solution of 3-oxauracil at 37·1 ± 0·1°C in the following solutions: *1* 0·1M-HCl, *2* 0·1M citrate at pH 5·24, *3* 0·067M phosphate at pH 7·15, *4* 0·067M phosphate at pH 7·56, *5* 0·1M borate at pH 10·38, *6* 0·1M borate at pH 11·36.

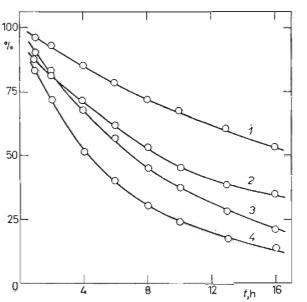

FIG. 4

3-Oxauracil Hydrolysis in Some Incubation Media

$1{\cdot}77 \cdot 10^{-3}$M 3-oxauracil at 37·1 ± 0·1°C in: *1* 0·01M phosphate at pH 7·58, *2* 0·02M Tris-HCl at pH 7·61, *3* 0·1M phosphate at pH 7·36, *4* synthetic cultivation medium without glucose at pH 6·98.

* The primary product of hydrolysis of 3-oxauracil is probably β-carboxylaminoacrylic acid which is rapidly decomposed to formylacetic acid. The β-carboxylaminoacrylic acid was obtained by Rinkes[2] in the form of an unstable aqueous solution by a Hoffmann degradation of maleic monoamide.

Since 3-oxauracil is decomposed relatively rapidly even in a neutral medium it was necessary to establish its course of decomposition in media applied for biological experiments. For this purpose the hydrolysis of 3-oxauracil was examined in a series of buffers and in a synthetic cultivation medium at 37°C. The time course of hydrolysis is shown in Fig. 4. Reaction mixtures with phosphate were shown to contain only unreacted 3-oxauracil after a 16-hour hydrolysis. Since the presence of formylacetic acid could not be demonstrated chromatographically (in an acid medium it is decarboxylated), the content of formylacetic acid was determined in the reaction mixtures after 16 h of hydrolysis, spectrophotometrically (Table I).

It was found during hydrolysis of 3-oxauracil in Tris-HCl that its decomposition has a more complex character. Chromatography showed not only unreacted 3-oxauracil (R_F 0·58) but also a UV-absorbing compound (R_F 0·32; UV spectrum in 0·1M-HCl: λ_{max} 259 nm; in 0·1M-NaOH: λ_{max} 252·5 nm) which is formed in a reaction of 3-oxauracil with Tris. The structure of this compound as well as the reaction of 3-oxauracil with other amines and amino acids will be studied in the future.

It is assumed that the biological effects of 3-oxauracil are not caused by the spontaneously formed degradation products of this compound. The biological effect is brought about either by the compound itself or by compounds formed from 3-oxauracil by enzyme catalysis.

Effect of 3-*Oxauracil on the Biosynthesis of DNA, RNA and Proteins*

3-Oxauracil added to growing *E. coli* at $5 . 10^{-4}$M concentration stops immediately the synthesis of DNA (Fig.5) was well as of RNA (Fig. 6). The inhibition of synthesis of both types of nucleic acids is of equal intensity. Figs 5 and 6 show that the inhibition

TABLE I

Per Cent Content of 3-Oxauracil and Formylacetic Acid after 16 h of 3-Oxauracil Hydrolysis in Neutral Buffers at 37°C

Buffer	pH	% 3-Oxauracil	% Formylacetic acid[a]
0·01M Phosphate	7·58	54·2	26·1
0·1M Phosphate	7·36	21·1	26·7
Synthetic growth medium	6·98	15·3	30·1
0·02M Tris-HCl	7·61	39·1	11·8

[a] The per cent content of formylacetic acid was calculated from a tentative value of absorbance of this acid in 0·05M-NaOH (ε 17 860 at 258 nm) which was derived on the basis of assuming the 3-oxauracil hydrolysis to yield at 25°C in 0·05M-NaOH quantitatively formylacetic acid.

of DNA and RNA synthesis can be substantially relieved by uridine added together with 3-oxauracil.

As could be expected, the inhibition of RNA synthesis is reflected in decreased protein formation (Fig. 7). In agreement with the reversal of RNA biosynthesis by uridine, protein synthesis is also clearly increased if 3-oxauracil is counteracted by uridine. The inhibition of protein synthesis (as well as its reversal by uridine) sets in later than the inhibition of DNA and RNA synthesis which justifies our view on the secondary effect of 3-oxauracil on the process of translation.

We thought it probable that the inhibition of DNA and RNA biosynthesis is caused by one of the following mechanisms: *1*) 3-Oxauracil interferes as such with the biosynthesis of nucleic acid precursors; *2*) lethal synthesis gives rise to compounds from 3-oxauracil such as inhibit the incorporation of pyrimidine nucleotides into nucleic acids; *3*) 3-oxauracil is incorporated into polynucleotides and the anomalous nucleic acids formed cannot fulfil their biological role.

In the present work we followed the possibility under *1*), *i.e.* interference of 3-oxauracil with enzymes catalyzing the formation of uridine 5'-monophosphate from aspartic acid.

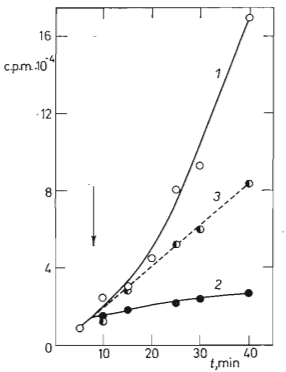

FIG. 5

Inhibition of DNA Synthesis by 3-Oxauracil

1 No inhibitor, *2* 3-oxauracil, *3* 3-oxauracil plus uridine. Experimental conditions in the text. The arrow shows the time of adding 3-oxauracil or of 3-oxauracil + uridine.

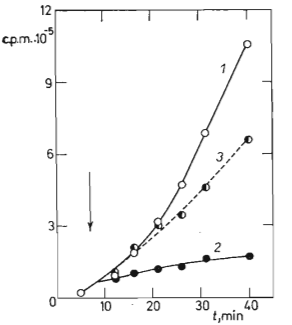

FIG. 6

Inhibition of RNA Synthesis by 3-Oxauracil

See legend to Fig. 5.

Interconversions of Dihydroorotic and Orotic Acids in the Presence of 3-Oxauracil

During incubation of labelled dihydroorotic acid with 3-oxauracil in the presence of NAD and a cell-free extract of *E. coli* B (using a 15-hour culture) a steady state between ureidosuccinic acid, dihydroorotic acid and orotic acid is attained within 15 min. 3-Oxauracil present in the incubation mixture had no effect on the metabolic conversions of dihydroorotic acid an hence it apparently does not block either dihydroorotase or dihydroorotate dehydrogenase.

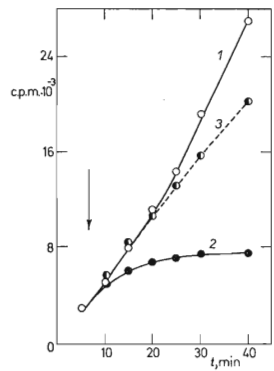

FIG. 7
Inhibition of Protein Synthesis by 3-Oxauracil
See legend to Fig. 5.

To check the sensitivity of the enzyme system used toward inhibitors of pyrimidine metabolism we carried out a parallel experiment with the well-known inhibitor of dihydroorotate dehydrogenase, *viz.* dihydro-5-azaorotic acid[10]. In equimolar concentrations of the inhibitor and substrate a powerful inhibition of orotic acid formation was observed.

In another series of experiments we followed the anabolism of labelled orotic acid in the presence pf 5-phosphoribosyl-1-pyrophosphate and of cell-free extracts from *E. coli* B with and without 3-oxauracil; a 10 min preincubation of 3-oxauracil in the absence of orotic acid was applied to allow for any phosphoribosylation of 3-oxauracil.

Within 15 min of incubation practically all the orotic acid was anabolized. A cell-free extract from cells harvested during the stationary phase of growth catalyzed the conversion of orotic acid to uridine 5′-monophosphate and a small amount of orotidine 5′-monophosphate. The cell-free extracts from cells harvested at the beginning of the logarithmic phase of growth catalyzed the formation of uridine 5′-monophosphate, of higher uridine phosphates and of orotidine 5′-monophosphate.

The formation of these compounds and their mutual ratio were not affected by 3-oxauracil even at a concentration ten times higher than that of substrate. These findings indicate that 3-oxauracil or rather its phosphoribosyl derivative, does not inhibit either orotidine 5'-phosphate pyrophosphorylase or orotidine 5'-phosphate decarboxylase.

Application of a cell-free extract from a "young" culture was justified by a parallel study where 3-oxauracil was replaced by 6-azauracil, a growth inhibitor of *E. coli* B (ref.[11]). In an incubation mixture with the "old" extract no effect of 6-azauracil on orotic acid metabolism was observed. When, however, the extract from "young" cells was used, orotidine 5'-monophosphate was preferentially formed, the formation of uridine 5'-monophosphate being markedly inhibited while the higher uridine phosphates were present only in trace amounts.

Antagonism of Pyrimidine Compound Precursors and 3-Oxauracil in Intact Cells

The above-mentioned negative findings of the effect of 3-oxauracil on enzyme systems catalyzing the biosynthesis of uridine 5'-monophosphate were further checked by growth experiments with intact cells of *E. coli*. Table II indicates that the growth-inhibiting effect of 3-oxauracil can be relieved only by preformed pyrimidines.

TABLE II

Antagonism of 3-Oxauracil and of Pyrimidine Compound Precursors in *Escherichia coli* B
Concentration of inhibitor as well as of precursors was always 10^{-4} M.

Addition to medium	Growth, %
0	100
3-Oxauracil	0
3-Oxauracil + aspartate	0
3-Oxauracil + ureidosuccinate	0
3-Oxauracil + dihydroorotate	0
3-Oxauracil + orotate	2
3-Oxauracil + uracil	92
3-Oxauracil + uridine	100

A number of precursors of uridine 5'-monophosphate *via de novo* synthesis from aspartic acid, showed no effect on the inhibition by 3-oxauracil. The results are in agreement with the above negative findings in cell-free extracts.

The study shows further that the observed inhibition of DNA and RNA biosynthesis by 3-oxauracil is not caused by an interaction of this antimetabolite with enzymes catalyzing the synthesis of uridine 5'-monophosphate.

A further examination of the molecular mechanism of the antibacterial effects of 3-oxauracil is now under way.

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