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MECHANISM OF INHIBITORY EFFECTS OF 2,3-DIHYDRO-1,3-6H-OXAZINE-2,6-DIONE (3-OXAURACIL)

BIOSYNTHESIS OF 3-OXAURIDINE PHOSPHATES IN INTACT CELLS AND CELL-FREE EXTRACTS OF *ESCHERICHIA COLI*

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Abstract—The supernatant fraction of *Escherichia coli* cells treated with [^{14}C]-2,3-dihydro-1,3-6H-oxazine-2,6-dione ([^{14}C]-3-oxauracil) contains the principal portion of radioactivity in the low molecular weight subfraction. The dominant component of this subfraction is a compound with the properties of 3-oxauridine 5′-monophosphate. The compound is also formed from 3-oxauracil and 5-phosphoribosyl 1-pyrophosphate on incubation with the cell-free extract of *E. coli*. If this incubation mixture is enriched with adenosine 5′-triphosphate, two further compounds are formed, their properties corresponding to 3-oxauridine 5′-di- and -triphosphates. Treatment of the compound possessing properties of 3-oxauridine 5′-triphosphate with snake venom yielded a non-nucleotide compound with a lower chromatographic mobility than authentic 3-oxauridine. This compound appears to be a ribonucleoside with an open oxazine ring.

It was observed in 1972 [1] that 2,3-dihydro-1,3-6H-oxazine-2,6-dione (3-oxauracil), prepared for the first time in 1927 by Rinkes [2], will inhibit growth of *Escherichia coli* B in a mineral medium with glucose. At the same time, but independently of our results, Kuhar *et al.* [3] described inhibitory effects of 3-oxauracil on leukemia L1210 cells *in vitro* as well as on the growth of *E. coli*. The third group studying, at that time, the inhibitory properties of 3-oxauracil was headed by Heidelberger (see the opening section in Ref. 4). 3-Oxauracil was found to be an effective cancerostatic agent [5, 6] for various experimental neoplasms, possessing clear immunosuppressive properties [7].

We have found that 3-oxauracil in an aqueous medium is decomposed at a rate dependent on the pH of the medium, giving rise to formylacetic acid which is further transformed to acetaldehyde. The hydrolytic cleavage of the oxazine ring of 3-oxauracil is connected with loss of biological activity; the final degradation products are integrated into the ongoing metabolism [8].

The therapeutically interesting properties of some halogen derivatives of natural pyrimidines led us to prepare some halogen derivatives of 3-oxauracil [9] and to study their inhibitory properties [10]. We found that 5-chloro-, 5-bromo- and 5-iodo-3-oxauracil act as potent inhibitors of the growth of *E. coli* B. The halogen derivatives of 3-oxauracil in aqueous solution are less stable than 3-oxauracil itself, being decomposed to the corresponding halogen acetaldehydes, which react with cell proteins, and are thus responsible for the inhibitory effects of the original compounds [10]; 5-fluoro-3-oxauracil is also inhibitory [11]. It is possible that even here the final inhibitor is fluoroacetaldehyde.

A ribonucleoside [4] and a deoxyribonucleoside [12] of 3-oxauracil with pronounced inhibitory properties were also prepared.

Since the discovery of the inhibitory effects of 3-oxauracil, we have been interested in their molecular mechanisms. In the first paper on 3-oxauracil [1], we described the observation that the antibacterial effect is completely eliminated by the presence of preformed pyrimidines in the medium. In mice with L1210 leukemia, a suitable combination of 3-oxauracil with uridine suppressed the toxic effects of 3-oxauracil but retained the cancerostatic effect of this antimetabolite [7].

In a later study, we established that, in a cell-free extract of *E. coli*, 3-oxauracil did not inhibit the conversion of orotic acid to uridine 5′-monophosphate even when preincubated with 5-phosphoribosyl 1-pyrophosphate and adenosine 5′-triphosphate [8]. On the other hand, we established in the same study [8] that addition of 3-oxauracil to the incubation medium caused an almost immediate inhibition of the biosynthesis of DNA and RNA in intact cells of *E. coli* B; protein synthesis was inhibited only after a certain time lag. 3-Oxauracil and its 5-halogen derivatives showed no mutagenic effects [10].

It can be concluded from the existing knowledge of molecular mechanisms of 3-oxauracil that the key question now is whether enzymic conversion of this antimetabolite to anomalous nucleotides takes place in target cells and tissues. The present paper attempts to contribute to the elucidation of this problem, which is highly complicated by the instability of 3-oxauracil and its derivatives.

MATERIALS AND METHODS

Analysis of the supernatant fraction of sonicated cells grown in the presence of [2,6-^{14}C]-2,3-dihydro-1,3-6H-oxazine-2,6-dione ([^{14}C]-3-oxauracil). Escherichia coli B cells were exposed for 30 min to [^{14}C]-3-oxauracil and then washed with 0.02 M Tris–HCl buffer, pH 7.5, and disintegrated ultrasonically (Ultrasonic disintegrator MSE 100 W, 22 kc, ampli-

tude 8, for 2 min at 0°). The sonicated mixture was centrifuged at 30,000 *g* and the supernatant fraction was chromatographed on a column of Sephadex G-200 (51 × 1.5 cm, 6 ml/hr, 3.35-ml fractions, 0.02 M Tris–HCl, pH 7.5, 20°). The individual fractions were analyzed for A_{280}, total radioactivity, radioactivity of the ice-cold trichloroacetic acid (TCA) precipitate and radioactivity of the hot TCA precipitate (15 min, 100°). Fraction No. 26, containing the radioactive component soluble in 5% TCA, was evaporated and analyzed by paper chromatography in a system of isobutyric acid–1 M NH_4OH–0.1 M EDTA of pH 8.2 (50:30:0.8).

Analysis of the acid-soluble pool of E. coli *B after uptake of* [^{14}C]-*3-oxauracil.* [^{14}C]-3-Oxauracil (2.5 μCi/μmole) at a concentration of 10 μg/ml was added to a culture of *E. coli* B in the exponential phase of growth in a synthetic medium [13]. At the moment of adding the analog the culture contained 3.5×10^8 cells/ml. After a 30-min incubation at 37°, the culture was cooled rapidly in an ice bath and filtered rapidly through a Sartorius membrane filter (0.45-μm pores). Cells on the filter were washed with ice-cold water and suspended in ice-cold 5% TCA. Extraction of cells with 5% TCA proceeded for 20 min at 0°. The precipitate was removed by filtration through a membrane filter and the TCA extract was freed of TCA by a repeated extraction with diethyl ether. After evaporation *in vacuo* the concentrated solution was analyzed by paper chromatography in ethyl acetate–water–formic acid (60:35:5). The region at the start was eluted with water and chromatographed on a thin layer of polyethylenimine cellulose (Polygram, Cel 300 PEI/UV$_{254}$, Macherey-Nagel) in 1.2 M LiCl–0.07 M CH_3COOH [14].

Reaction of 3-oxauracil with 5-phosphoribosyl 1-pyrophosphate in a cell-free extract of E. coli *B.* To study the biosynthesis of 5′-ribonucleotides of 3-oxauracil *in vitro*, we used the dialyzed 30,000 *g* supernatant fraction of *E. coli* B (Ultrasonic disintegrator MSE, 100 W, 22 kc, amplitude 8, for 2 min at 0° in 0.02 M Tris–HCl, pH 7.5) prepared from cells at the beginning of the exponential phase of growth. The incubation mixture contained in 1 ml: 12.5 μmoles Tris–HCl buffer, pH 7.5, 6.25 μmoles $MgCl_2$, 0.625 μmole [^{14}C]-3-oxauracil (2.5 μCi/μmole), 2.5 μmoles 5-phosphoribosyl 1-pyrophosphate, 2.5 μmoles ATP, and 9 mg of total protein of the 30,000 *g* supernatant fraction of *E. coli* B.

After 30 min of incubation at 37° the nonreacted 3-oxauracil was separated by paper chromatography in 1-butanol–acetic acid–water (10:1:3) where the nucleotides remain at the start. The start region containing 10 per cent of total radioactivity was eluted with water and analyzed by paper electrophoresis in 0.02 M sodium citrate, pH 3.6. Paper strips corresponding to the mobility of nucleoside mono- and triphosphates were eluted with water, the eluates were concentrated *in vacuo*, and the residues were dissolved in 0.02 M Tris–HCl, pH 7.4, and treated with 50 μg alkaline phosphatase (*E. coli*) or 100 μg of snake venom (*Crotalus adamanteus*) for 1 hr at 37°. The reaction products were analyzed by paper chromatography in 1-butanol–acetic acid–water (10:1:3).

*2,3-Dihydro-1,3-6*H*-oxazine-2,6-dione.* 3-Oxauracil was prepared according to Washburne *et al.* [15]. [^{14}C]-2,3-Dihydro-1,3-6H-oxazine-2,6-dione of molar activity 1.94 mCi/m-mole was supplied by the Institute for Research, Production and Uses of Radioisotopes (Prague).

*2,3-Dihydro-3-β-ribofuranosyl-1,3-6*H*-oxazine-2,6-dione.* This compound and its 5′-phosphate were prepared by the following procedures. A stirred mixture of 2,3,5-tri-*O*-acetyl-D-ribofuranosyl bromide (6.8 g) and 2,3-dihydro-3-trimethylsilyl-1,3-6H-oxazine-2,6-dione [15] (4.0 g) in 60 ml acetonitrile were combined with mercuric chloride (5.8 g) added in parts over 15 min at room temperature. After 12 hr of standing at room temperature the acetonitrile was distilled *in vacuo* and the residue was dissolved in 300 ml chloroform, washed with three portions of a 10% aqueous solution of potassium iodide containing 5% acetic acid (150 ml total) and three times with water, dried (Na_2SO_4), and evaporated *in vacuo*. Chromatography on a column of silica gel in benzene–ethyl acetate (1:1) yielded 3.2 g of syrupy 2,3-dihydro-3-(2,3,5-tri-*O*-acetyl-D-ribofuranosyl)-1,3-6H-oxazine-2,6-dione; $[\alpha]_D^{25} + 21.46°$ (c 0.42; ethyl acetate). Calc. for $C_{15}H_{17}NO_{10}$: C, 48.52; H, 4.61; N, 3.77. Found: C, 48.12; H, 4.90; N, 3.28.

The tri-*O*-acetyl derivative from the preceding experiment (3.2 g) was dissolved in 300 ml of 0.1 M HCl in methanol and, after 20 hr of standing at room temperature, the mixture was treated with Dowex 1 (acetate), filtered and evaporated *in vacuo*. The residue was chromatographed on a column of silica gel in ethyl acetate–acetone–methanol–water (14:1:0.5:0.5). Rechromatography of the syrupy residue (900 mg) on a column of microcrystalline cellulose in 1-butanol–acetic acid–water (20:3:7) yielded 350 mg of chromatographically homogeneous syrup which was diluted with ethyl acetate and left to stand for several days at 0°. The solution crystallized to 90 mg of 2,3-dihydro-3-β-D-ribofuranosyl-1,3-6H-oxazine-2,6-dione, melting at 116–118° (ethyl acetate); λ_{max} (H_2O) 267 nm (log ε 3.96). CD spectrum (water) $[\theta]_{237.5} - 3790$, $[\theta]_{272} + 6740$. ^{1}H-N.m.r. spectrum (100 MHz, in DMSO-d_6): 3.60 (2H, m, H-5′a and H-5′b, $J_{5'a,5'b}$ 10 Hz, $J_{5'a,4}$ 2.6 Hz, $J_{5'b,4}$ 2.6 Hz), 3.80 to 4.00 (2H, m, H-3′ and H-4′), 4.03 (1H, q, H-2′, $J_{2',1'}$ 4.0 Hz, $J_{2',3'}$ 6.8 Hz), 5.61 (1H, d, H-1′, $J_{1',2'}$ 4.0 Hz), 5.68 (1H, d, H-5, $J_{5,4}$ 8.5 Hz), 8.13 (1H, d, H-4, $J_{4,5}$ 8.5 Hz). Consumption of periodic acid at 20° (acetate buffer, pH 4.7): 0.96 mole in 5 min, 1.04 moles in 1 hr. Calc. for $C_9H_{11}NO_7$: C, 44.09; H, 4.52; N, 5.71. Found: C, 44.29; H, 4.56; N, 5.68. On the basis of the ^{1}H-n.m.r. spectrum it may be concluded that the sample prepared here is identical with that prepared by Chwang *et al.* [4] as a syrup, using a different method. Reaction of 3-oxauridine (90 mg) with acetone (1 ml) and 2,2-dimethoxypropane (1.5 ml) under catalysis with traces of hydrochloric acid yielded 49.0 mg of 2′,3′-*O*-isopropylidene-3-oxauridine melting at 141–143°. ^{1}H-N.m.r. spectrum (100 MHz, in DMSO-d_6): 1.31 (3H, s, CH_3), 1.51 (3H, s, CH_3), 3.64 (2H, m, 2H-5′), 4.22 (1H, m, H-4′, $J_{4',3'}$ 3.0 Hz), 4.78 (1H, dd, H-3′, $J_{3',4'}$ 3.0 Hz, $J_{3',2'}$ 6.1 Hz), 4.95 (1H, dd, H-2′, $J_{2',3'}$ 6.1 Hz, $J_{2',1'}$ 2.25 Hz), 5.12 (1H, t, OH), 5.72 (1H, d, H-5, $J_{5,4}$ 8.0 Hz), 5.75 (1H, d, H-1′, $J_{1',2'}$ 2.25 Hz), 7.83 (1H, d, H-4, $J_{4,5}$ 8.0 Hz). Mass spectrum: *m/e* 270 $(M-15)^+$. Ultraviolet spectrum (in ethanol): λ_{max} 266 nm (log ε 3.89).

2′,3′-*O*-Isopropylidene-3-oxauridine (14 mg) from the preceding experiment was dissolved in acetonitrile (1.0 ml), cooled to −40°, and pyrophosphoryl chloride (100 mg) was added. After 10 min, the reaction mixture was removed from the cooling bath and left to stand for 2 hr at room temperature. After cooling to −10°, it was poured into 20 ml of ice-cold water. After 1 hr of standing at room temperature, the oxazine derivatives were adsorbed on 200 mg of active charcoal. After 10 min the charcoal was filtered and washed with 50 ml water to neutral reaction. The product was desorbed by washing on a filter with 5% aqueous pyridine (50 ml). The eluate was evaporated *in vacuo* and purified by paper electrophoresis in acetic acid–pyridine–water (3:14:983) on Whatman 3MM filter paper at 2000 V. The absorbing zone of phosphate was eluted with 10% formic acid and the eluate was filtered through a short column of Dowex 50 (H^+) to remove the pyridine. The yield of 3-oxauridine 5′-phosphate, referred to 2′,3′-*O*-isopropylidine-3-oxauridine and estimated spectrophotometrically, amounted to 11 per cent. The product was characterized by its u.v. spectrum (λ_{max} 265 nm), by its thin-layer chromatography on polyethylenimine cellulose, and by its electrophoretic mobility in a citrate buffer (pH 4.0). The compound gives a positive reaction for organically bound phosphate and for vicinal diols.

RESULTS AND DISCUSSION

To establish the nature of the radioactive compounds formed in growing cells of *E. coli* exposed to [^{14}C]-3-oxauracil, we ultrasonicated the cells and separated the supernatant fraction of the mixture on a column of Sephadex G-200. Figure 1 shows the main portion of radioactivity to be present in fractions 21–29, corresponding to low molecular weight compounds of the bacterial pool. The fraction of radioactivity in the high molecular fractions was not analyzed in detail, but it was found that extraction with cold or hot 5% TCA will not liberate low molecular weight compounds from these high molecular weight fractions. Fraction No. 26 (Fig. 1), containing the maximum of low molecular weight radioactive compounds, was chromatographed on paper. The dominant component of the analyzed fraction was a compound with mobility of 3-oxauridine 5′-monophosphate (Fig. 2).

Analogous results were obtained by a chromatographic analysis of the TCA extract of intact cells of *E. coli*, exposed to [^{14}C]-3-oxauracil. The TCA extract was first subjected to paper chromatography in a system where the nucleotide fraction remains at the start. After developing the chromatogram, the start region was found to contain 80 per cent of the total TCA-extract radioactivity, while 20 per cent of the total moved like 3-oxauracil. After elution of the compounds from the start region and subsequent chromatography of the eluate on a thin layer of polyethylenimine cellulose, the principal fraction of radioactivity was localized in a zone moving like 3-oxauridine 5′-monophosphate (Fig. 3).

The finding of a radioactive compound with nucleoside monophosphate character formed in *E. coli* cells in the presence of 3-oxauracil supported our working hypothesis [8] that 3-oxauracil is anabolized in cells of *E. coli* to anomalous nucleotides.

The fate of [^{14}C]-3-oxauracil in a cell-free extract of *E. coli* was then followed in the presence of 5-phosphoribosyl 1-pyrophosphate, both with and without the addition of adenosine 5′-triphosphate. After 30 min,

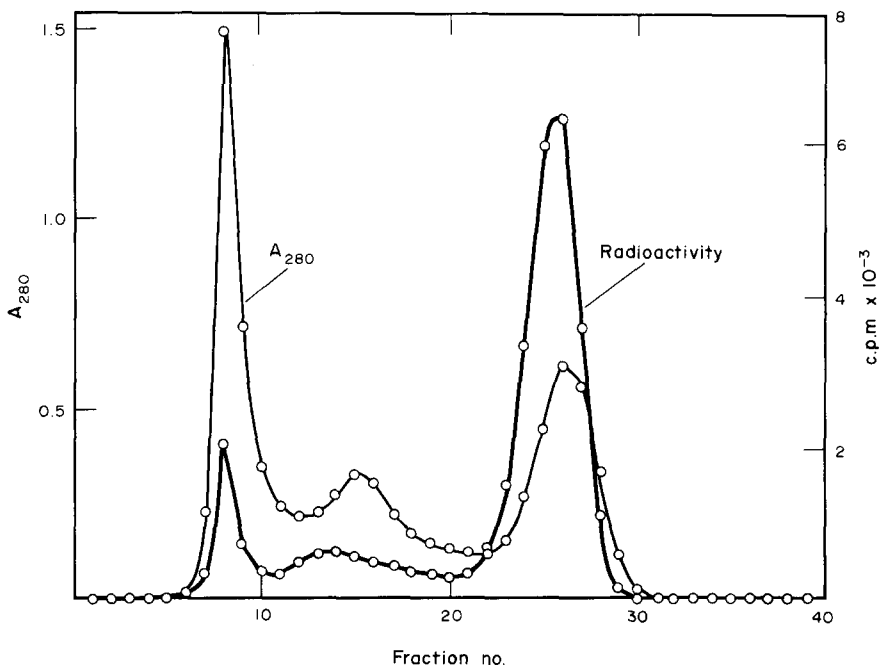


Fig. 1. Sephadex G-200 gel filtration of the supernatant fraction of sonicated cells of *E. coli* grown in the presence of [^{14}C]-3-oxauracil.

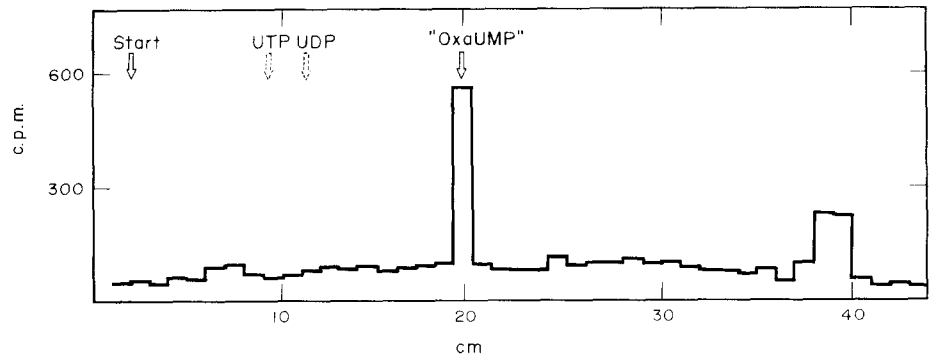


Fig. 2. Chromatography of fraction No. 26 from Sephadex G-200 gel filtration (see Fig. 1) in isobutyric acid–1 M NH_4OH–0.1 M EDTA (50:30:0.8). The mobility of synthetic 3-oxauridine 5′-monophosphate (3-OxaUMP) is indicated. For orientation, the mobilities of uridine 5′-diphosphate (UDP) and -triphosphate (UTP) are given.

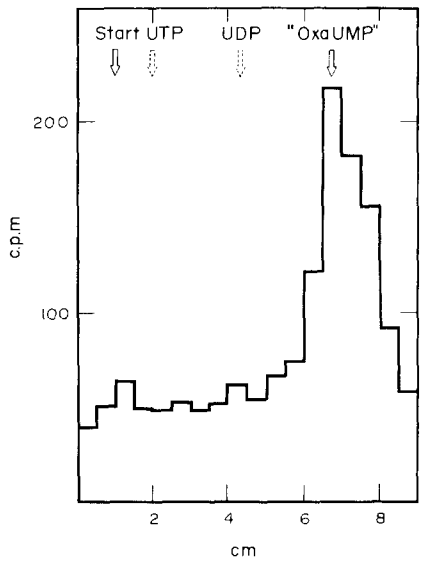
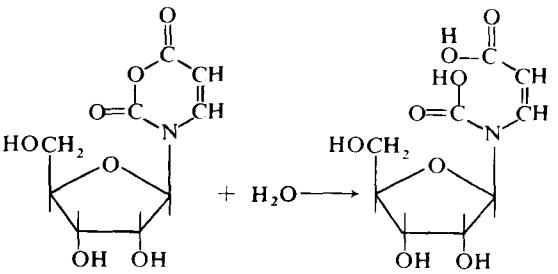


Fig. 3. Chromatography of the acid-soluble pool of cells of *E. coli* exposed to [^{14}C]-3-oxauracil. The chromatography was performed on a PEI-cellulose plate in 1.2 M LiCl–0.07 M CH_3COOH at 20°. The mobility of synthetic 3-oxauridine 5′-monophosphate (3-OxaUMP) is indicated. For orientation, the mobilities of uridine 5′-diphosphate (UDP) and -triphosphate (UTP) are given.

the two samples were freed of nonreacted 3-oxauracil by paper chromatography. The nucleotide fraction at the chromatogram start was eluted and separated by high-voltage paper electrophoresis. The distribution of radioactivity on the electrophoretograms is shown in Fig. 4. In the absence of adenosine 5′-triphosphate, the dominating radioactive compound in the nucleotide fraction has a mobility resembling that of 3-oxauridine 5′-monophosphate. If adenosine 5′-triphosphate is present during incubation, a pronounced change takes place in the distribution of radioactivity on the electrophoreogram: the compound with 3-oxauridine 5′-monophosphate mobility practically disappeared while two radioactive zones appeared, one moving like uridine 5′-diphosphate, the other like uridine 5′-triphosphate. This difference may be explained by the fact that 3-oxauridine 5′-monophosphate, formed from 3-oxauracil and 5-phosphoribosyl 1-pyrophosphate under catalysis of phosphoribosyl transferase in the cell-free extract, is further phosphorylated enzymically in the presence of adenosine 5′-triphosphate, giving rise to 3-oxauridine 5′-di- and triphosphates.

The radioactive zone with a mobility like that of uridine 5′-triphosphate was eluted from the electrophoreogram and treated with snake venom. The digest was chromatographed on paper (Fig. 5). The compound of nucleotide nature (remaining at the start in this chromatographic system) completely disappeared. However, at the site of 3-oxauridine where maximum radioactivity was expected, a low radioactivity was actually found. The principal portion of radioactivity was bound to a non-nucleotide compound moving during chromatography much more slowly than 3-oxauridine.

On the basis of the comparison of the half-lives of 3-oxauracil (600 min), its ribonucleoside (90 min) and ribonucleotide (200 min), it can be deduced that 3-oxauridine, formed during the enzymatic dephosphorylation of the 3-oxauridine 5′-phosphates, is quickly hydrolyzed to a ribonucleoside with an opened oxazine ring:



This degradation also proceeds during the isolation procedure and the analytical separation. It is not excluded that the oxazine ring might be opened to some extent also in 3-oxauridine 5′-phosphates. Nevertheless, 3-oxauridine 5′-monophosphate seems to be the primary anabolite of 3-oxauracil. The role of 3-oxauridine 5′-phosphates in the molecular mechanism of the inhibitory effects of 3-oxauracil will be studied in further work.

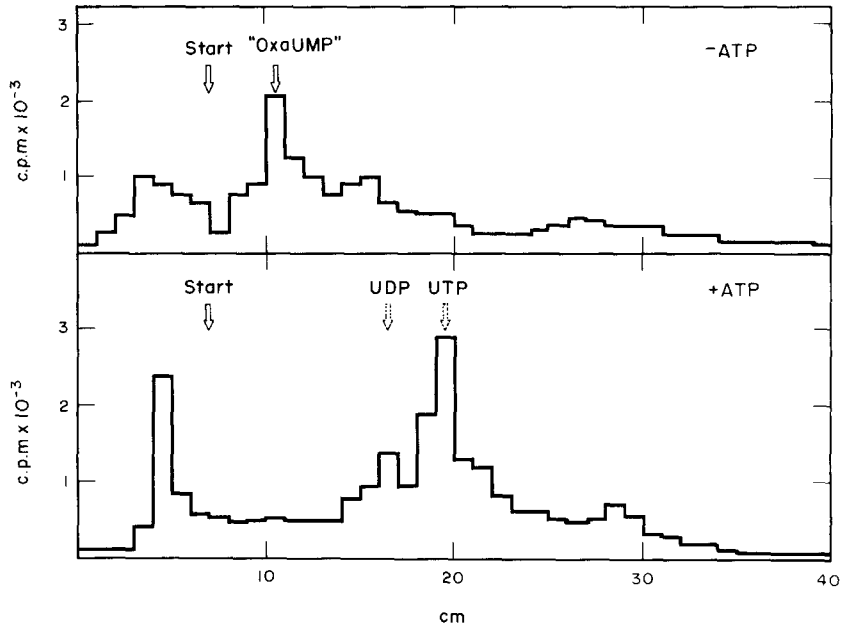


Fig. 4. *In vitro* biosynthesis of 3-oxauracil ribonucleotides in a cell-free extract of *E. coli*. Paper electrophoresis of the reaction products was performed in 0.02 M citrate buffer, pH 3.6, for 1 hr 45 min. The mobility of synthetic 3-oxauridine 5′-monophosphate (3-OxaUMP) is indicated. For orientation, the mobilities of uridine 5′-diphosphate (UDP) and -triphosphate (UTP) are given.

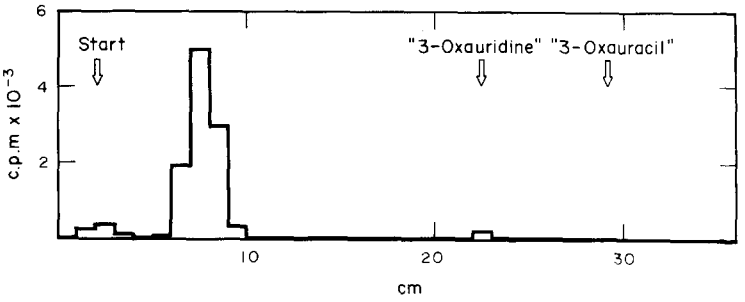


Fig. 5. Rattlesnake venom splitting of *in vitro* synthesized [^{14}C]-3-oxauridine 5′-triphosphate (see Fig. 4). The chromatogram was developed in 1-butanol–acetic acid–water (10:1:3).

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