INHIBITION OF DNA SYNTHESIS IN *Escherichia coli* BY 1-(β-D-RIBOFURANOSYL)-2-PYRIMIDONE

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1-(β-D-Ribofuranosyl)-2-pyrimidone (*I*) strongly inhibits the division of *Escherichia coli* cells. It penetrates through the membrane only to a small extent. The pool of *E. coli* K 12 was found to contain at most 3% of the applied concentration of *I*. The mechanism of inhibition of DNA synthesis in the presence of *I* is discussed. Study of the template activity of GpUpPyR showed that compound *I* cannot replace uridine in the triplet codons.

In connection with studying the biochemical behaviour of analogues of pyridine ribonucleosides we took up the preparation and the properties of 1-(β-D-ribofuranosyl)-2-pyrimidone (*I*) and of related compounds. Compound *I*** is a near analogue of uridine from which it differs by the absence of the N^3-H group and of the 4-keto group (and hence cannot form either actively or passively hydrogen bonds). It was hence of interest to examine the possible efficiency of compound *I* in an *in vivo* system. For this purpose, we chose the study of bacteriostatic activity of the compound toward *E.coli*. In the course of this work, Øyen[1] published some results on the same compound. It follows from his study that, in agreement with our findings, *I* inhibits DNA synthesis while the synthesis of RNA and of proteins is not affected.

EXPERIMENTAL

Chemicals. 2-Pyrimidone (*II*), 1-(β-D-ribofuranosyl)-2-pyrimidone (*I*), 1-(β-L-ribofuranosyl)-2-pyrimidone (*III*), 1-(β-D-ribopyranosyl)-2-pyrimidone (*IV*) and 3-(β-D-ribofuranosyl)-4-pyrimidone (*V*) were synthesized according to ref.[2]. 1-(β-D-Ribofuranosyl)-2-pyrimidone 5'-phosphate (*VIa*) was prepared according to ref.[3], 1-(β-D-ribofuranosyl)-(2)-pyrimidone 5'-diphosphate (*VIb*) according to a commonly used procedure[5].

UpPyR was prepared by a reaction of U > p with nucleoside *I* in the presence of pancreatic ribonuclease[6]. Uridine 2',3'-cyclic phosphate (triethylammonium salt) (50 μmol) and nucleoside *I*

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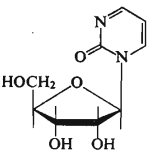
** Abbreviations used: Py, (2)-pyrimidone (*II*), PyR, 1-(β-D-ribofuranosyl)-(2)-pyrimidone (*I*), pPyR, 1-(β-D-ribofuranosyl)-(2)-pyrimidone 5'-monophosphate (*VIa*). Other abbreviations are to be found in ref.[4].

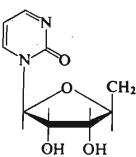
(150 μmol) in 150 μl 50% pyridine were incubated for 20 h at 0°C with 50 μg pancreatic ribonuclease (Calbiochem) and the mixture was chromatographed in system S 3. The band of the product (R_F 0·20) was eluted with water (5 ml) and the product was freeze-dried. The yield was 25%. The product is split by pancreatic ribonuclease to uridine 3′-phosphate and to nucleoside *I* in an equimolar ratio. GpUpPyR and pGpUpPyR were prepared by the reaction of G > p and pG > p, respectively, with UpPyR in the presence of T1 ribonuclease under standard conditions[6]. The yield was 5% and 1·5%, respectively. The compounds are split by T1 ribonuclease to Gp (or pGp) and UpPyR. The labelled compounds used, uridine-[^{14}C] (190 μCi/μmol), 2′-deoxy-thymidine-[2-^{14}C] (43 μCi/μmol), CDP-[^{14}C] (50 μCi/μmol), valine-[^{14}C] (146 μCi/μmol), were obtained from the Institute for Research, Production and Application of Radioisotopes in Prague. 1-(β-D-Ribofuranosyl)-2-pyrimidone-[2-^{14}C] (3·3 μCi/μmol) was prepared in analogy to a procedure described elsewhere[7]. Orthophosphate-[^{32}P] was carrier-free, from Isocomerz, Berlin-Buch, German Democratic Republic.

Cultivation of bacteria. E. coli B was grown in a mineral medium with glucose[8], *E. coli* K 12 Hfr in the same medium containing 0·2% casamino acids (Difco), *B. subtilis* SMYW was grown in a medium according to Spizizen[9]. The cultures were labelled with ^{32}P in a low-phosphate medium[10]. The uptake of radioactive precursors by the bacterial cells or incorporation into nucleic acids was followed with the aid of filtration through membrane filters[11]. The bacterial pool was extracted with cold 5% trichloroacetic acid. This was then removed from the extract by shaking with ether. The extract obtained was concentrated by freeze-drying and then analyzed. DNA synthesis was followed by incorporation of orthophosphate-[^{32}P] into the fraction stable for 16 h in 0·33M-KOH at 37°C. Cell-free extract *I* from *E. coli* B was prepared from a bacterial suspension by sonication on a MSE 100 W ultrasonic disintegrator at 24 kc, amplitude 6·5, for 2 min at 0°C, in 0·02M Tris-HCl at pH 7·4. The disintegrated cells were centrifuged at 30 000 *g* and the super-



I

II



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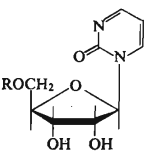
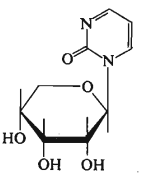
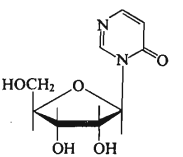




IV



V

VIa, R = —P(O)(OH)$_2$

VIb, R = —P(O)(OH)—O—P(O)(OH)$_2$

natant was desalted on a column of Sephadex G-25 (coarse) at 4°C in the same buffer. Cell-free extract *II* from *E. coli* B with nucleoside reductase activity (E. C. 1.8) was prepared according to Reichard[12]. Enzyme hydrolysis and phosphorylation *in vitro* were studied in 50 μl of a mixture containing 4 μmol Tris-HCl at pH 7·55, 0·4 μmol $MgCl_2.6\,H_2O$, 0·125 μmol [^{14}C]-(*I*) or uridine-[^{14}C], and eventually, 0·5 μmol ATP, and cell-free extract *I* containing 800 μg protein. The reaction proceeded for 30 min at 37°C. After termination of the reaction the mixture was placed on Whatman No 3 MM and developed in the descending direction in the S 1 system. The system with nucleoside diphosphate reductase activity contained in 250 μl: 3 μmol Tris-HCl at pH 7·85, 1 μmol ATP, 3 μmol $MgCl_2.6\,H_2O$, 1 μmol $NADPH_2$, 0·09 μmol CDP-[^{14}C], 1·88 μmol diphosphate *VIb*, and cell-free extract *II* containing 2 mg protein. The reaction mixture was incubated for 25 min at 37°C and placed on a strip of Whatman No 3MM. The reaction products were identified by descending chromatography in a S 2 system and rechromatographed in the same system using Whatman No 1. *Radioactivity* was determined in a Packard 3375 liquid scintillation counter.

Binding assay. The binding of [^{14}C]-Val-tRNA to ribosomes was measured by the membrane filtration technique[13]. The reaction mixture contained in a final volume of 0·05 ml: 2·5 μmol Tris-acetate at pH 7·2, 5 μmol ammonium acetate, 1 μmol magnesium acetate, 2·5 A_{260} units of ribosomes[14], 0·55 A_{260} unit of tRNA acylated[14] with 18·2 pmol valine-[^{14}C] and 0·10 A_{260} unit of the oligonucleotide. After incubation (20 min at 24°C) the mixtures were diluted with 3 ml of the same buffer, filtered through Millipore filters, washed with four 5 ml portions of cold buffer and the filters were dried. The radioactivity was determined in a proportional methane-flow counter of Frieseke-Hoepfner. All experiments were done in duplicate and averaged.

Chromatography and electrophoresis. System S 1: 1-butanol–acetic acid–water (10 : 1 : 3), system S 2: 99% ethanol–1M ammonium acetate (pH 3·8)–0·1M disodium ethylenediaminetetraacetate[15] (pH 8·2) (75 : 29 : 1); system S 3: 2-propanol–concentrated NH_4OH–water (7 : 1 : 2). Compounds *I* and *VIa* were identified by electrophoresis on Whatman No 3 MM in 0·05M triethylammonium borate at pH 7·5. The electrophoresis was performed in an apparatus of Markham and Smith[16], for 80 min at a potential gradient of 40 V/cm. The distribution of radioactivity on the chromatographic paper was examined on a Frieseke-Hoepfner scanner PH 452.

RESULTS AND DISCUSSION

Nucleoside *I* is an inhibitor of bacterial growth. During stationary cultivation the absorbance of the cultures at 575 nm was decreased on addition of 10 μg of the com-

TABLE I

Cell Division in Normal and in Nucleoside (*I*)-Inhibited Culture of *Escherichia coli* Experimental details see text. Total count [$ml^{-1} . 10^{-8}$].

Time min	*E. coli* B		*E. coli* K 12 Hfr	
	control	PyR	control	PyR
0	7·2	7·2	5·4	5·4
90	16·8	6·2	9·6	3·4

pound per ml medium using *E. coli* B by 85–90%, using *E. coli* K 12 Hfr by 90–95%, and using *B.subtilis* SMYW by 90%. The efficiency is thus much greater than that reported by Øyen[3]. In contrast with stationary cultivation, the addition of the same amount of analogue to an exponentially growing culture of *E.coli* B showed no significant difference in absorbance between the inhibited culture and the control

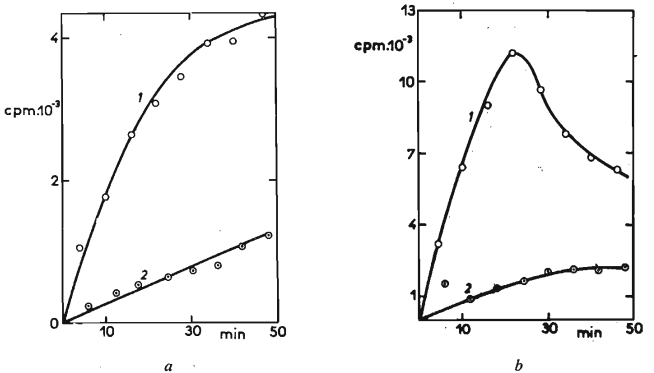


FIG. 1

Penetration of Nucleoside *I* into *E. coli*

Concentration of *I*-[2-^{14}C]: 30 μg/ml (0·44 μCi/ml). Penetration into a *E. coli* B, *b E. coli* K 12 Hfr, *1* ^{14}C counts in whole cells, *2* ^{14}C counts insoluble in cold 5% trichloroacetic acid.

TABLE II

Products of Hydrolysis and Phosphorylation of Nucleoside *I* in a Cell-free System of *E. coli* B

Amounts of products formed are given in nmol. For details see the text.

Substrate	Addition	Py	PyR	pPyR
PyR-[^{14}C]	—	79	46	—
	ATP	78	41	4
		U	Urd	UMP
Urd[^{14}C]	—	31	93	—
	ATP	41	57	25
	ATP, PyR	40	52	30

experiment. However, data of Table I indicate that the total number of bacteria per ml culture after 90 min of exposure to the analogue (10 μg/ml) was not increased in an exponentially growing culture. Compound *I* thus strongly inhibits the division of bacterial cells. The cells are much larger in the presence of analogue and have a filamentous appearance.

Analogues *III*—*V* are completely bacteriostatically ineffective under the same conditions. Similarly inefficient is also 2-pyrimidone (*II*). The inefficiency of the L-enantiomer *III* may be attributed among other things also to the fact that the L-ribonucleosides do not penetrate the cell membrane of *E. coli*[17].

Penetration of nucleoside *I* into *E. coli* cells was examined in an exponentially growing culture. *I*-[2-^{14}C] was added at a culture density corresponding to A_{575} of 0·2 to a final concentration of 30 μg/ml and the incorporation was examined for 50 min. The results shown in Fig. 1*a*,*b* demonstrate that *I* is taken up by the cells of *E. coli* B much less than by *E. coli* K 12. The highest penetration into *E. coli* K 12 was attained between 20th and 25th min, amounting to at most 3% of the applied amount (30 μg/ml). The uptake of the nucleoside *I* by *E. coli* is accompanied by a hydrolysis of the nucleosidic bond and by an accumulation of 2-pyrimidone (*II*) in the medium (Fig. 2). The main part (75—90%) of the radioactivity inside the cell was found in the pool, *i.e.* in a fraction soluble in cold 5% trichloroacetic acid (Fig. 1*a*,*b*). The nucleosidic bond of *I* is hydrolyzed also *in vitro* using a cell-free extract of *E. coli* B, both in the presence of ATP and without it (Table II).

In the experiment reported in Table II the stability of the nucleosidic linkage in *I* was compared with that of uridine. The results indicate that *I* is less stable toward enzyme hydrolysis of the nucleoside bond than uridine. In an *in vitro* system containing ATP no significant phosphorylation of *I* to its monophosphate *VIa* was achieved (Table II) as compared with uridine, which suggests that *I* is not a suitable substrate for nucleoside kinase *in vitro*.

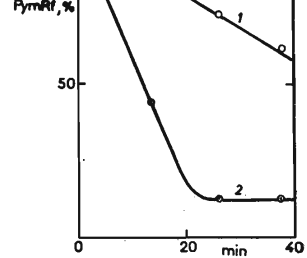


FIG. 2
Stability of Nucleoside *I* in the Medium
1 E. coli B, *2 E. coli* K 12 Hfr

The pool extracted from *E. coli* B after 50-min incorporation of *I*-[2-^{14}C] was shown by chromatography in S 1 to contain *I* (26%), *II* and an unidentified compound (29%), and *VIa* (45%). After cleavage of *VIa* with alkaline phosphatase, electrophoresis in borate buffer showed the greater part of radioactivity to be present in nucleoside *I*. Hence it follows that *in vivo* the nucleoside *I* which entered into the bacterial cells is partly phosphorylated to the 5'-monophosphate *VIa*. A small amount of nucleoside *I* which can penetrate into the cells of *E. coli* markedly inhibits the synthesis of DNA (Fig. 3*a*). The inhibition is relieved completely by adding an excess of uridine together with the analogue (Fig. 3*a*). An excess of 2'-deoxyadenosine does not compete with the inhibition of DNA synthesis. On the other hand, *I* does not inhibit for the first 15 min the incorporation of 2'-deoxythymidine into *E. coli* K 12 (Fig. 3*b*). If 2'-deoxyadenosine is added to the medium together with *I*, 2'-deoxyadenosine being known to inhibit thymidine phosphorylase *in vivo*[18], the incorporation of 2'-deoxythymidine is not inhibited at all (Fig. 3*b*) although under these conditions the synthesis of DNA is inhibited (Fig. 3*b*, curve *4*). Nucleoside *I* thus apparently facilitates the incorporation of 2'-deoxythymidine similarly to a number of other nucleosides[19] by inhibiting thymidine phosphorylase *in vivo*. After a 15 min incorporation a greater part of *I*

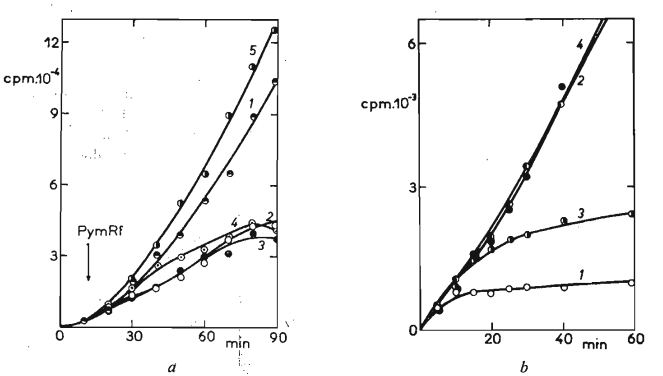


FIG. 3

Inhibition of DNA Synthesis in *E. coli* K 12 Hfr by Nucleoside *I*

a ^{32}P counts stable in 0·33M-KOH for 16 h. The additions were made to parts of the culture after 11 min (referred to 1 ml): *1* no addition, *2* 30 μg *I*, *3* 60 μg *I* and *4* 60 μg *I* and 200 μg 2'-deoxyadenosine, *5* 60 μg *I* and 200 μg uridine. *b* Incorporation od 2'-deoxythymidine-[2-^{14}C] (3 μg/ml; 0·1 μCi/ml). The additions were made to parts of the culture at time zero (referred to 1 ml): *1* no addition, *2* 2'-deoxyadenosine 200 μg, *3* 30 μg *I*, *4* 2'-deoxyadenosine 200 μg and 30 μg *I*.

in the medium has been converted to 2-pyrimidone (*II*) (Fig. 2) and the incorporation of 2'-deoxythymidine hence ceases (Fig. 3*b*). In another experiment the effect of *I* or of its 5'-diphosphate (*VIb*) upon the reduction of cytidine 5'-diphosphate was studied *in vivo* in a system according to Reichard[12]. The results demonstrate that in the presence of the diphosphate *VIb* 38 nmol dCDP and 4·5 nmol dCMP are formed as compared with 31 nmol dCDP, 3·5 nmol dCMP and 4·3 nmol dCyd in a control experiment. Diphosphate *VIb* hence has no significant effect on the *in vitro* reduction of cytidine 5'-diphosphate.

Our further results support the observations shown in the literature[3] that compound *I* has no effect on the synthesis of RNA and only slightly inhibits the incorporation of leucine-[^{14}C] into an exponentially growing culture of *E. coli* B. The incorporation of *I* into mRNA should result in an inhibition of protein synthesis *in vivo*. This assumption was confirmed by a study of the template activity of triplets GpUpPyR and pGpUpPyR upon the binding of Val-tRNA to the ribosomes. The presence of nucleoside *I* in the third position of the codon results in a complete loss of template activity according to:

Template	None	GpUpU	GpUpPyR	pGpUpPyR
pmol	0·58	2·40	0·62	0·64
Δ pmol	—	1·82	0·04	0·06

Compound *I* thus cannot replace uridine in the triplet codons. Since the incorporation of leucine (the codons of which contain uridine) is not markedly affected by the presence of *I* in the medium the result provides indirect proof that compound *I* is probably not incorporated into mRNA.

Nucleoside *I* penetrates, even if in small amounts, into the cells of *E. coli* and markedly suppresses bacterial growth. The division of bacterial cells is inhibited most probably in consequence to an inhibition of synthesis of DNA. Nucleoside *I* in contact with a bacterial cell is relatively rapidly hydrolyzed to the base *II* which is bacteriostatically ineffective and accumulates in the medium. The pool of the bacterial cells was found to contain the monophosphate *VIa*. It cannot thus be excluded that the above-mentioned hydrolysis of the nucleosidic bond is due to a previous enzyme phosphorylation of nucleoside *I* to *VIa*. Since compound *I* does not inhibit the reduction of cytidine 5'-diphosphate and since its effect on DNA synthesis is limited by the uptake of 2'-deoxythymidine from the medium it is probable that nucleoside *I* inhibits *in vivo* the synthesis of 2'-deoxythymidine 5'-monophosphate.

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