SUBSTRATE SPECIFICITY OF BACTERIAL THYMIDYLATE-SYNTHETASE

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Structural requirements of bacterial (*E. coli*) thymidylate-synthetase have been investigated on analogues of 2'-deoxyuridine 5'-phosphate. In interactions with thymidylate-synthetase, the following conditions must be fulfilled by the analogue: *1*) presence of a 5'-phosphate group capable for the formation of a dianion; *2*) presence of a free hydroxyl at position 5' in the *ribo* configuration; *3*) presence of a pyrimidine base capable for the interaction with the enzyme; the base must contain a nitrogen atom at position 3 and must be free of substituents at positions 3 and 4; *4*) the conformation of the nucleoside moiety must be *anti*; *5*) the mutual distance and spatial orientation of the bonding center for the phosphate and the region involved in the interaction with the heterocyclic base is strictly defined and highly sensitive to any change; *6*) in the case of the methylation reaction, the substrate must also contain a 5,6-double bond suitably activated by conjugation with a group at position 4 and lacking substituents at positions 5 and 6. Analogues of dUMP have been prepared by a chemical or enzymatical phosphorylation of the corresponding nucleosides. The preparation of 3'-O-benzoyl-2'-deoxyuridine, 3'-O-methyl-2'-deoxyuridine, 1-(2-deoxy-β-D-xylofuranosyl)uracil, and its 3'-O-benzoyl derivative has been reported.

Thymidylate-synthetase constitutes one of the key enzymes in the DNA metabolism; its role consists in catalysis of the transfer of the one-carbon fragment ("methylation reaction") from $N^{5,10}$-methylene-L-tetrahydrofolate into the position 5 of the pyrimidine ring of 2'-deoxyuridine 5'-phosphate. This reaction represents the main path in the synthesis of 2'-deoxythymidine and its nucleotides *de novo* in living cells. The pure enzyme has been recently isolated from various tissues or organisms and the mechanism of its action has been investigated[1-3].

The requirements of this enzyme towards the donor as well as acceptor component have been investigated with the principal aim to find specific inhibitors in the latter case. In this connection, a special attention has been paid to 5-fluoro-2'-deoxyuridine 5'-phosphate, the irreversible enzyme inhibitor, attached by a covalent bond to position 6 of the heterocyclic base[3]. In the case of 5-trifluoromethyl-2'-deoxyuridine 5'-phosphate, a similar mechanism has been postulated. From some other inhibitors reported in the literature, the activity of 5-mercapto[4,5], 5-formyl[6], and 5-hydroxymethyl[7] analogues of 2'-deoxyuridine 5'-phosphate and the activity of 2'-deoxypseudouridine 5'-phosphate[8] should be mentioned.

Most investigations in the earlier work on the specificity of thymidylate-synthetase have been performed with the nucleoside derivatives only and the partially purified enzyme preparation containing in addition to thymidylate-synthetase also thymidine-kinase which simultaneously acted as a phosphorylation factor. In such an approach

it is assumed that the modified nucleoside is a substrate for the kinase; the effect of the structural change on the interaction with thymidylate-synthetase could otherwise hardly manifest itself. The effect of modifications at all the three parts (phosphate, sugar, heterocyclic base) of the substrate molecule has not been so far investigated.

To avoid complications in the work with nucleosides alone, synthetic nucleotides and their analogues have been used in the present investigation along with a purified preparation of thymidylate-synthetase *E. coli* B. Not only the inhibitory activity of the analogue on the methylation of 2′-deoxyuridine 5′-phosphate has been examined, but also the substrate activity of uracil derivatives has been checked by the radioactivity determination of the product resulting from the analogue in the presence of [^{14}C]formaldehyde. This technique eliminates any uncertainty at the coincidence of the inhibitory and substrate activity, and makes possible to separate the enzyme requirements on the interaction with the analogue from requirements concerning the enzyme-catalysed methylation reaction.

The nucleoside 5′-phosphates modified in the heterocyclic or sugar moiety have been prepared from the corresponding nucleosides mostly by a direct 5′-phosphorylation with phosphorus oxychloride in triethyl phosphate[9] (method *A*). This method fails when the adjacent 3′-hydroxylic function is of the *xylo* configuration, *i.e.*, when situated *cis* with respect to the 5′-hydroxymethyl group; in this case, the reaction affords exclusively the corresponding 3′,5′-cyclic phosphate, resistant towards the acidic or alkaline hydrolysis. In such cases the corresponding 3′-O-benzoyl derivatives have been phosphorylated under the above conditions and the protecting benzoyl group removed by alkaline hydrolysis as it may be exemplified on the synthesis of 1-(2-deoxy-β-D-xylofuranosyl)uracil 5′-phosphate* (*XVI*):

dUrd → Tr-dUrd → Tr-dUrd-OMe → Tr-dXyloUra-OBz →
→ dXyloUra-OBz → pdXyloUra

3′-O-Methyl-2′-deoxyuridine 5′-phosphate (*XIX*) was prepared by phosphorylation of the parent nucleoside, obtained from 5′-O-trityl-2′-deoxycytidine by methylation at position 3′ with dimethyl sulfate in alkaline medium, detritylation, and finally deamination of 3′-O-methyl-2′-deoxycytidine:

dCyd → Tr-dCyd → Tr-dCyd-OMe → dCyd-OMe → dUrd-OMe

When the direct phosphorylation is not successful, the alternative method consists in the transfer of phosphoric acid residue from phenyl phosphate under catalysis of the carrot phosphotransferase[10] (method *B*):

* Abbreviations: Tr, trityl; Ms, methanesulfonyl; dXyloUra, 1-(2-deoxy-β-D-xylofuranosyl)uracil; Nuc, nucleoside.

$$\text{Nuc} + C_6H_5O\text{—}P(O)(OH)_2 \rightarrow \text{pNuc} + C_6H_5OH$$

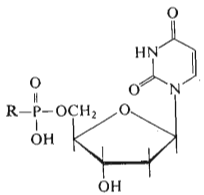
This specific method affords 5′-nucleotides in fair yields.

The 2′-deoxyuridine 3′-phosphate analogues with the modified phosphoric acid residue were prepared from 3′-O-benzoyl-2′-deoxyuridine by the reported procedures[11–15]. The latter nucleoside derivative is obtained from 5′-O-trityl-2′-deoxyuridine by benzoylation with benzoyl cyanide and detritylation of the intermediate:

$$\text{Tr-dUrd} \rightarrow \text{Tr-dUrd-OBz} \rightarrow \text{dUrd-OBz}$$

The analogues of 2′-deoxyuridine 5′-phosphate (dUMP) of the present study may be divided into four groups. The first group comprises analogues modified in the phosphoric acid residue at position 5′ of 2′-deoxyuridine while the nucleoside moiety remains untouched. None of these compounds (*I* – *VI*) shows any activity as a substrate or as an inhibitor of the methylation reaction of dUMP. In these compounds, the phosphoric acid residue is modified by a formal replacement of one of the hydroxyls by the hydrogen atom (compound *I*), methyl group (*II*), hydroxymethyl group (*III*), amino group (*IV*) or methoxy group (*V*). The common feature of these compounds is the loss of the dissociable group. The steric effect of the substituent on the interaction with the bonding center may be excluded since the 5′-phosphite *I*, the substituent of which shows minimum steric requirements, is neither substrate nor inhibitor. The structural requirement of thymidylate-synthetase thus appears to consist in the presence of a phosphate group capable to form a dianion. The number of dissociable groups on the phosphorus atom does not represent the single determining factor; as it may be inferred from the inactivity of the 5′-diphosphate *VI*, the position of these groups on the phosphorus atom must be α.

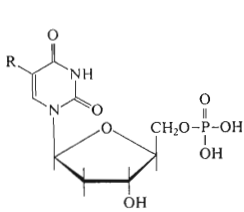
In the second group of dUMP analogues, the sugar moiety has been modified. This group also does not contain any substance which would be active as the enzyme



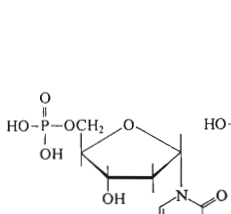
I; R = H
II; R = CH_3
III; R = CH_2OH
IV; R = NH_2
V; R = OCH_3
VI; R = O—P(O)(OH)$_2$

inhibitor or substrate. It may be inferred from the inactivity of enantiomeric L-nucleotides *VII* and *VIII* in both functions (2′-deoxy-D-thymidine 5′-phosphate of the D-series is inhibitor of the product type) that in the bond of the substrate or inhibitor molecule to the enzyme, three different regions are involved including the chiral nucleoside portion of the molecule[16]. When the phosphate residue is assumed as one of these regions, there are at disposal two additional centers for an exact orientation of the analogue molecule with respect to the protein molecule. It may be assumed on the basis of analogy with some other cases[16] that the heterocyclic moiety of the molecule represents one of these centers; in this moiety, moreover, the catalysed reaction takes place. In nucleosides of the L-series, however, this heterocyclic base cannot be oriented in such a manner to make possible a bonding or cooperative interaction which would increase the stability or readiness of the complex formation between the enzyme and the substrate or inhibitor. The same situation may be observed in the case of the α-nucleotide *IX*, the uracil ring of which is situated on the opposite side of the sugar ring plane (with respect to the 5′-substituent); a simultaneous interaction of the phosphate group and the heterocycle is thus again impossible.

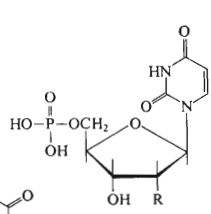
Introduction of a substituent into position 2′ of the sugar moiety, *e.g.*, a halogen atom, an amino group, an azido group or an alkoxyl of the *ribo* configuration with compounds *X*–*XIV*, or a hydroxyl of the *ribo*[17] or *arabino* configuration with compound *XV*, results in a complete loss of the characteristic properties of the parent substance. The 2′-deoxyribofuranose ring thus constitutes the fundamental requirement of the substrate structure; a steric influence of the substitution may be hardly expected since the 2′-fluoro-2′-deoxy group is almost isosteric with methylene group (*cf.* compound *X*). Another obvious structural requirement consists in the presence



VII; R = H
VIII; R = CH_3



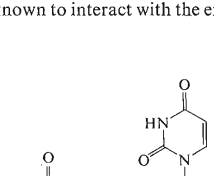
IX



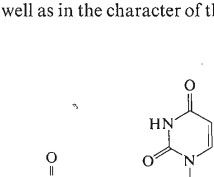
X; R = F
XI; R = Cl
XII; R = NH_2
XIII; R = N_3
XIV; R = OCH_3

of the hydroxy group. This group must be localised at position 3′ since the isomeric 3′-deoxyuridine 5′-phosphate (*XVIII*) with interchanged positions of the deoxy group and the hydroxy group, is completely inactive in both processes. The hydroxy group at position 3′ must be free (*cf.* compound *XIX*) and in the *ribo* configuration; compound *XVI*, the 3′-hydroxyl of which assumes the *xylo* configuration, is inactive.

In the 2-deoxy-α-L-lyxofuranosyl derivative *XVII*, the 3′-hydroxyl assumes the due orientation with respect to the uracil residue but the phosphate group is oriented *cis* with respect to the 3′-hydroxyl. This configurational change at position 4′ brings about a deep change in the mutual distance of the two centers (phosphate and base) which are known to interact with the enzyme as well as in the character of the phospho-



XV

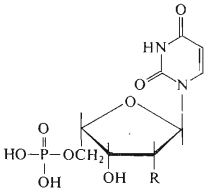


XVI; R = H
XX; R = OH

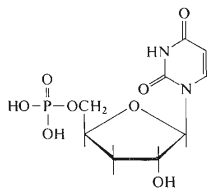
rus atom surroundings which are involved in the bond formation. Consequently, the derivative *XVII* is inactive both as substrate and as inhibitor. The structural requirements of the enzyme are also not fulfilled by compound *XXIII*, the aliphatic analogue of dUMP; the structure of the sugar ring thus also represents one of the essential features of the substrate[18].

The above observations are in accordance with those made in the third group of dUMP analogues. Thus, the isomeric nucleotide, namely, 2′-deoxyuridine 3′-phosphate (*XXIV*) and its derivatives *XXV*–*XXVII* substituted at position 2′ by a hydroxy, amino or azido group, are neither enzyme substrates nor inhibitors due to the changed distance between the phosphorus atom and the heterocyclic base. The 3′, 5′-diphosphate *XXVIII* is also inactive; in this compound, however, the hydroxy group at position 3′ is not free (*cf.* compound *XIX*).

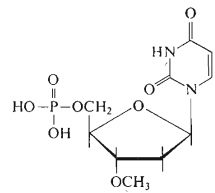
In this connection, it was of interest to examine the behaviour of short 2′-deoxyuridine-containing oligonucleotides with respect to thymidylate-synthetase since the methylation reaction might occur on the level of some other precursors or degradation products of DNA or *vice versa*, such oligonucleotides might interfere in the reaction proceding simultaneously on the level of a monomer. The dinucleotides d(pUpU) and



XVII, R = H
XXI, R = OH



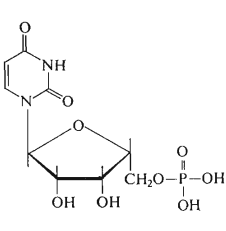
XVIII



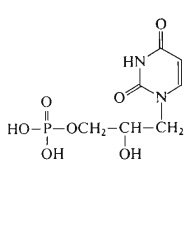
XIX

d(pUpC) are, however, completely inactive in both these directions. This combination also eliminates the influence of the 2′-deoxyuridine residue at the 3′-end on the activity of the molecule. The two parent dinucleoside phosphates *XXXI* and *XXXII* lacking the 5′-terminal phosphoric acid residue, are also inactive. The above mentioned oligonucleotides may, however, be considered as 2′-deoxyuridine 5′-phosphate esters or its 3′-O-substituted derivatives. As shown above, any of these modifications of the dUMP molecule results in a loss of activity. The methylation of 2′-deoxyuridine derivatives to 2′-deoxythymidine derivatives *in vivo* thus appears to proceed exclusively on the level of the monomer, *i.e.*, 2′-deoxyuridine 5′-phosphate.

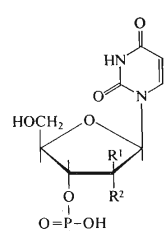
The last group of dUMP analogues is represented by compounds modified in the heterocyclic moiety while the 2′-deoxyribofuranosyl 5′-phosphate portion of the molecule remains intact (Table I). This group also contains most information on the specificity of thymidylate-synthetase. The replacement of uracil by cytosine is known to



XXII



XXIII



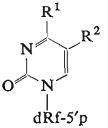
XXVIII; pdUrd-p
XXIX; d(pUpU)
XXX; d(pUpC)
XXXI; d(UpU)
XXXII; d(UpC)

XXIV; $R^1 = R^2 = H$
XXV; $R^1 = OH$, $R^2 = H$
XXVI; $R^1 = H$, $R^2 = NH_2$
XXVII; $R^1 = H$, $R^2 = N_3$

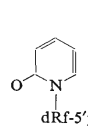
result in an inactive molecule of 2′-deoxycytidine 5′-phosphate[19]. On the other hand, the 4-hydroxylamino derivative, N^4-hydroxy-2′-deoxycytidine 5′-phosphate, is claimed as a very weak competitive enzyme inhibitor[20]. Since the latter difference might be due to the amino–imino tautomerism of the heterocyclic moiety, the behaviour of mono- and disubstituted dCMP derivatives *XXXIII* and *XXXIV* has been examined. Both these compounds are inactive. The hydrophilic character of the 2-hydroxyethyl substituent on the nitrogen atom at position 4 of the ring excludes hydrophobity to be responsible for the behaviour of compound *XXXIII*. It is also not allowed to modify the substrate by replacement of the oxygen atom at position 4 of the uracil ring by a sulfur atom, probably because of the increased electronegativity or increased steric requirements of the substituent.

It is of interest that the interaction with the enzyme does not require the presence of the 4-oxo group as it may be inferred from our earlier observations on the strong competitive enzyme inhibitory character of the 2-pyrimidinone 2′-deoxyribofuranosyl derivative *XXXVI* (ref.[21]). The corresponding 5-methyl derivative *XXXVII* is also a competitive inhibitor of the methylation reaction, though to a lesser extent. As it may be inferred from these observations, the 4-oxo group is not necessary in the interaction with the enzyme and the free N^3-H group is not necessarily required by the inhibitor structure. The failure of the inhibitory activity with 4-substituted derivatives *XXXIII*–*XXXV* might be more likely ascribed to the steric effect of a bulky substituent.

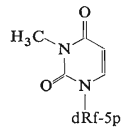
In contrast to the inhibitor *XXXVI*, the 2-pyridinone 2′-deoxyribofuranosyl derivative *XXXVIII* is neither substrate nor inhibitor of the enzyme. Contrary to the former compound, the latter derivative contains at position 3 of the heterocyclic ring a methine group instead of the nitrogen atom. The presence of a N^3-atom in the heterocyclic system is thus a condition *sine qua non* for the inhibitory activity of the particular analogue. In the case of N^3-methyl-2′-deoxyuridine 5′-phosphate (*XXXIX*), the methylation of the uracil nucleus in dMP extinguishes any activity towards the en-



XXXIII; $R^1 = NH(CH_2CH_2OH)$, $R^2 = H$
XXXIV; $R^1 = N(CH_3)_2$, $R^2 = H$
XXXV; $R^1 = SH$, $R = H$
XXXVI; $R^1 = R^2 = H$
XXXVII; $R^1 = H$, $R^2 = CH_3$



XXXVIII



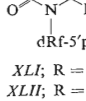
XXXIX

zyme and proves the sensitivity of this position with respect to introduction of a bulky substituent. As mentioned in the preceding paragraph, the free N^3-H group (compound *XXXVI*) is not required by the inhibitor structure.

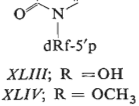
An indifferent behaviour towards the enzyme has been also observed with the 6-azauracil and 2′-deoxyorotidine derivatives *XL* and *XLI*, resp. Similarly to other cases [16], the increased acidity of the heterocyclic moiety in both these derivatives might be the reason of a decreased affinity since this portion of molecule interacts more likely with some other strongly basic center of the protein molecule than with that region originally responsible for the interaction with the heterocyclic moiety. Compounds *XL*, *XLI*, and the 6-methyl-2′-deoxyuridine derivative *XLII* (also inactive in both processes) have in common a conformational change in comparison with the normal *anti* conformation of pyrimidine derivatives: the considerable angle distorsion with the 6-azauracil derivative *XL* on the one hand and the reversal of conformation with the 6-methyl-2′-deoxyuridine derivative *XLII* on the other hand. A proper interaction with the heterocyclic base would require in the case of these substances a further energy to bring the base into a proper conformation; such a process would decrease the probability of the ES-complex formation. It is also necessary to take into account the explanation based on the assumed mechanism of the enzyme action[4] consisting in addition of the nucleophilic group of the enzyme to position 6 of the uracil 5,6-double bond, followed by an attack of the activated $N^{5,10}$-methylene-L--tetrahydrofolate at position 5 and elimination. Such a process might be of course affected or made impossible by substitution at position 6 or replacement of the methine group by the nitrogen atom. The inactivity of compounds *XL*–*XLII* as enzyme substrates is most likely explained by the last mentioned alternative; the inhibitor inactivity (and thus the impossibility to form a complex with the enzyme), however, might be ascribed to any arguments stated above.

The 5-formyluracil analogue of dUMP is a noncompetitive thymidylate-synthetase inhibitor[6] as it has been mentioned above while 5-hydroxymethyl-2′-deoxyuridine

XL



XLI; R = COOH
XLII; R = CH_3



XLIII; R = OH
XLIV; R = OCH_3
XLV; R = OC_2H_5
XLVI; R = $OCH_2CH_2CH_3$
XLVII; R = $N(C_2H_5)_2$

In formulae *XXXIII* - *XLVII* dRf-5′p = 2′-deoxyribofuranosyl 5′-phosphate residue

5′-phosphate (*XLIII*) is claimed to act as a competitive inhibitor[7]. This conclusion has been now confirmed by our measurements. Not only compound *XLIII*, but also its alkoxy derivatives, such as 5-methoxymethyl, 5-ethoxymethyl, and 5-(1-propyloxy)methyl derivatives *XLIV*–*XLVI* are competitive enzyme inhibitors almost equally active as compound *XLIII*. Since the position 5 is occuppied, none of these compounds can be substrate in the methylation reaction. In contrast to these compounds, no activity has been observed in the case of 5-diethylaminomethyl-2′-deoxyuridine 5′-phosphate (*XLVII*). The 5-hydroxymethyl or 5-alkoxymethyl derivatives can probably act as inhibitors of the reaction product type (such as 2′-deoxythymidine 5′-phosphate); the steric substituent effect in the propyloxy derivative must then be lower than the effect of the bulky diethylamino group in the compound *XLVII*. In the case of the latter derivative, the strongly basic character of the diethylaminoethyl substituent of the uracil nucleus must be also taken into account and, *inter alia*, made responsible for the decreased probability of the complex formation with the enzyme.

The above mentioned results afforded some information on the structural requirements for the formation of a complex between the substrate and thymidylate-synthetase and for the inhibitory role of the substrate. None of the investigated series of substances except for the naturally occurring dUMP (Fig. 1) proved to be substrate for the actual enzyme-catalysed reaction, *i.e.*, methylation at position 5 of the uracil

TABLE I

Base-Modified 2′-Deoxyuridine 5′-Phosphate Analogues

Compound	Heterocyclic base	Inhibition	$K_i[M]$	K_i/K_m[a]
XXXIII	N^4-dimethylcytosine	0	—	—
XXXIV	N^4-(2-hydroxyethyl)cytosine	0	—	—
XXXV	4-thiouracil	0	—	—
XXXVI	2-pyrimidinone	86	$1{\cdot}5 \cdot 10^{-6}$	0·06
XXXVII	5-methyl-2-pyrimidinone	66	$3{\cdot}2 \cdot 10^{-6}$	0·13
XXXVIII	2-pyridone	0	—	—
XXXIX	N^3-methyluracil	0	—	—
XL	6-azauracil	0	—	—
XLI	6-carboxyuracil	0	—	—
XLII	6-methyluracil	0	—	—
XLIII	5-hydroxymethyluracil	38	$3{\cdot}9 \cdot 10^{-5}$	1·04
XLIV	5-methoxymethyluracil	34	$1{\cdot}4 \cdot 10^{-4}$	3·74
XLV	5-ethoxymethyluracil	28	$8{\cdot}8 \cdot 10^{-5}$	2·33
XLVI	5-(1-propyloxy)uracil	33	$2{\cdot}0 \cdot 10^{-4}$	5·33
XLVII	5-diethylaminomethyluracil	0	—	—

[a] $K_m = 3{\cdot}75 \cdot 10^{-5}$ M.

ring. From the group of inhibitors (Table I), the 5-substituted derivatives cannot serve as substrates; compound *XXXVI*, however, which is a strong competitive inhibitor, is also not methylated in spite of the free position 5 on the uracil ring[21]. This finding is in accordance with the recent idea on the mechanism of the enzyme action. On the basis of earlier observations and results obtained in the present work, the following conclusions can be drawn: for the interaction with thymidylate-synthetase, the dUMP analogue must fulfil the above-discussed structural requirements such as the presence of the 2'-deoxyribofuranose ring, the presence of a 3'-hydroxylic function in the *ribo* configuration, the *anti* conformation of the nucleoside, a phosphate residue capable of a twofold dissociation and exactly oriented towards the heterocyclic base, and finally, the presence of a heterocyclic pyrimidine base with a nitrogen atom at position 3 and free of bulky substituents at positions 3, 4, and 6. When these requirements are fulfilled, the substance is an enzyme inhibitor. These requirements are however insufficient for the realisation of the one-carbon-fragment transfer, *i.e.*, the actual thymidylate-synthetase catalysed reaction; an additional presence of an unsubstituted suitably activated[3,22,23] 5,6-double bond in the uracil ring is indispensable for an addition-elimination process with the participation of the enzyme and the donor. Such an activation can be accomplished by conjugation with the 4-oxo group but is insufficient in the 2-pyrimidinone derivative *XXXVI* where the 4-oxo group is lacking.

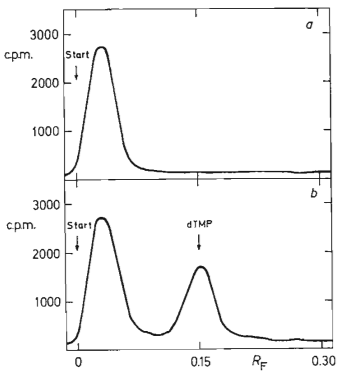


FIG. 1

Paper Chromatography (S_2) of the dUMP Methylation Mixture in the Presence of [^{14}C]Formaldehyde and *E. coli B* Thymidylate Synthetase

EXPERIMENTAL

Melting points were taken on a heated microscope stage (Kofler block) and are uncorrected. Unless stated otherwise, the solutions were taken down on a rotatory evaporator at 35°C/15 Torr and the drying of substances was performed over phosphorus pentoxide at room temperature and 0·05 Torr for 24 h.

Methods

Descending paper chromatography was performed on paper Whatman No 3 MM in the solvent system S_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2), and S_2, isobutyric acid–conc. aqueous ammonia–water (66 : 1·5 : 33). The paper electrophoresis was performed on paper Whatman No 3 MM at 30 V/cm (1 h) in the buffer solution 0·1M triethylammonium hydrogen carbonate (pH 7·5) using uridine 3′-phosphate as standard. Thymidylate-synthetase was isolated from *E. coli* and partially purified according to the method of Wahbe and Friedkin[24] to produce 9·5 μmol dTMP/mg of the protein per min. The thymidylate-synthetase test was performed as follows. The incubation mixture (total volume, 140 μl) contained 32·5 mM Tris-HCl buffer (pH 8·25), 16·2 mM magnesium chloride, 65 mM 2-mercaptoethanol, 5 mM EDTA, 9·75 mM formaldehyde, 0·325 mM $N^{5,10}$-methylene-L-tetrahydrofolate, $2{\cdot}2 \cdot 10^{-5}$M 2′-deoxyuridine 5′-phosphate (sodium salt, Calbiochem), [^{14}C] dUMP ($7 \cdot 10^3$ — $1{\cdot}4 \cdot 10^4$ dpm), and 10^{-6} to 10^{-5}M of the corresponding analogue. After the addition of the enzyme protein (35 μg), the mixture was incubated at 37°C for 10 min and the aliquots analysed in the solvent system S_2. The spots of dUMP and dTMP were cut out and the radioactivity determined on a liquid scintillation counter Packard. The methylation of the appropriate analogue was checked as follows: dUMP and [^{14}C] dUMP was omitted and replaced by [^{14}C]formaldehyde (25 μC_i/μmol, Centre d'energie nucleaire, Saclay) in the same final concentration of formaldehyde. The analysis was performed by chromatography in solvent systems S_1 and S_2, the chromatograms cut into 1 cm wide strips, and the radioactivity measured as above. For a typical course of the radioactivity separation see Fig. 1.

Materials

The modified nucleosides were prepared according to the references given in superscripts: L-2′--deoxyuridine[25], L-2′-deoxythymidine[25], 2′-deoxy-α-uridine [26], 2′-chloro-, 2′-azido-, and 2′-amino-2′-deoxyuridine[27], 2′-O-methyluridine[28], 1-(β-D-arabinofuranosyl)uracil[25], 1-(2-deoxy-β-D--xylofuranosyl)uracil[29], 1-(2-deoxy-α-L-lyxofuranosyl)uracil[30], 1-(α-D- and 1-(α-L-lyxofuranosyl)uracil[31], 1-(β-D-xylofuranosyl)uracil[32], 1-D-(2,3-dihydroxypropyl)uracil[33], 4-thio-2′-deoxyuridine[34], 2′-deoxy-6-azauridine[35], 5-fluoro-2′-deoxyuridine[36],5-ethoxymethyl-2′-deoxyuridine[25], 5-diethylaminomethyl-2′-deoxyuridine[36]. 3′-O-Acetyl-2′-deoxy-2′-fluorouridine was a gift of Dr W. Guschlbauer, Saclay, France; 3′-deoxyuridine was granted by Dr J. J. K. Novák of this Institute. Compounds *VII* and *VIII* were reported elsewhere[25]; compound *IX* was prepared according to ref.[26]; compounds *XII*, *XIII*, *XXVI*, and *XXVII* were obtained according to ref.[27]; compound *XXIII* is reported in ref.[33] Preparation of compounds *XXIX*—*XXXII* is given in ref.[39] 2′-Deoxyuridine 5′-phosphoramidate was obtained according to ref.[37] 2′-Deoxyuridine 3′-phosphate (*XXIV*) was prepared by phosphorylation of 5′-O-trityl-2′-deoxyuridine[38]. 2′-Deoxyuridine 5′-methyl phosphate[39] (*V*) and 2′-deoxyuridine 5′-diphosphate[37] were prepared by reported procedures. N^3-Methyl-2′-deoxyuridine 5′-phosphate (*XXXIX*) was prepared from dUMP by methylation with dimethylformamide dimethylacetal[40]. 1-(2-Deoxy-β-D-ribofuranosyl)-2-pyridinone 5′-phosphate was a gift of Prof. Ch. Tamm, Basel, Switzerland.

3′-O-Benzoyl-2′deoxyuridine

To a mixture of 5′-O-trityl-2′-deoxyuridine[41] (4·7 g; 10 mmol) and benzoyl cyanide (2·0 g; 15 mmol) in acetonitrile (25 ml) there was added portionwise with stirring triethylamine (2·5 ml) until the exothermic reaction set in. The mixture was then stirred at room temperature for 1 h, evaporated under diminished pressure, the residue heated in boiling ethanol (20 ml), and diluted with light petroleum (100 ml). The precipitate was separated, washed with light petroleum, refluxed in 80% aqueous acetic acid (50 ml) for 30 min, and the mixture evaporated under diminished pressure. The residue was coevaporated with three 50 ml portions of ethanol and then heated to the boiling point in ethanol (300 ml). After cooling, the solid was separated, washed with ethanol, and dried under diminished pressure. Yield, 2·0 g (75%) of 3′-O-benzoyl-2′-deoxyuridine, m.p. 224—226°C. For $C_{16}H_{16}N_2O_6$ (332·2) calculated: 57·82% C, 4·85% H, 8·43% N; found: 58·01% C, 4·92% H, 8·16% N.

3′-O-Methyl-2′-deoxyuridine

To a solution of 5′-O-trityl-2′-deoxycytidine[42] (2 mmol) and sodium hydroxide (20 g) in 70% aqueous methanol (500 ml) there was added dropwise over 1 h under stirring and cooling with ice-cold water dimethyl sulfate (42 ml) at the temperature below 50°C. The whole mixture was then stirred at room temperature for 1 h, the methanol evaporated, the residue diluted with water (200 ml), and extracted with three 100 ml portions of chloroform. The extract was washed with water (100 ml) and evaporated. The residue was refluxed in 80% aqueous acetic acid (100 ml) for 40 min and evaporated. The residue was diluted with water (100 ml) and washed with three 25 ml portions of ether. The aqueous phase was evaporated and the residue coevaporated with three 25 ml portions of water. The final residue was dissolved in a mixture of sodium hydrogen sulfite (6 g) and water (25 ml), the solution kept at room temperature overnight, and precipitated with a sufficient amount of saturated aqueous barium hydroxide. The barium sulfite was filtered off through Celite, the filtrate evaporated, and the residue chromatographed on two 40 × 16 × 0·4 cm layers of loose fluorescent-indicator-containing silica gel in the solvent system chloroform–ethanol (93 : 7). The band of the product was eluted with methanol (200 ml), the eluate evaporated, and the residue crystallised from water to afford 115 mg (24%) of 3′-O-methyl-2′-deoxyuridine, m.p. 148—149°C. For $C_{10}H_{14}N_2O_5$ (242·2) calculated: 49·58% C, 5·82% H, 11·56% N; found: 50·00% C, 5·90% H, 11·72% N.

1-(3-O-Benzoyl-2-deoxy-β-D-xylofuranosyl)uracil

A solution of 5′-O-trityl-2′-deoxyuridine (4 mmol) and methanesulfonyl chloride (2 ml) in pyridine (20 ml) was kept at room temperature overnight, diluted with water (5 ml), kept for 1 h more, diluted with additional water (200 ml), and extracted with three 50 ml portions of chloroform. The extract was washed with two 100 ml portions of dilute (1 : 10) hydrochloric acid, saturated aqueous sodium hydrogen carbonate (100 ml), water (50 ml), and dried over magnesium sulfate. Filtration and evaporation of the filtrate afforded a residue which was chromatographed on two layers of loose silica gel (see above) in the solvent mixture benzene–ethyl acetate (1 : 1). The product (R_F 0·35; Tr-dUrd, R_F 0·20) was eluted with methanol (200 ml), the eluate evapcrated, and the residue dried under diminished pressure. The residual 3′-O-methanesulfonyl-5′-O-trityl-2′-deoxyuridine (1·5 g; 68·5%) was then kept at room temperature in saturated methanolic ammonia (60 ml) overnight. The mixture was then evaporated, the residue refluxed for 2 h with 50% aqueous methanol (50 ml) and 1M methanolic sodium methoxide (10 ml), the mixture concentrated to the volume of about 10 ml, diluted with water (200 ml), and extracted with three 50 ml portions of

chloroform. The extracts were combined, washed with water (50 ml), dried over anhydrous magnesium sulfate, and evaporated. A mixture of the residue, acetonitrile (25 ml), and benzoyl cyanide (0·4 g; 3 mmol) was treated dropwise under stirring with triethylamine (1 ml) until the exothermic reaction set in. After 1 h at room temperature, the solution was evaporated, and the residue refluxed in 80% aqueous acetic acid (50 ml) for 1 h. The mixture was evaporated, the residue coevaporated with two 50 ml portions of ethanol, and chromatographed as above on two layers of loose silica gel in the solvent mixture ethanol–chloroform (2 : 98). The product (R_F 0·07) was eluted with methanol (200 ml), the eluate evaporated, and the residue crystallised from ethanol (10 ml), cyclohexane being added to the turbidity. Yield, 0·80 g (2·4 mmol; 60%, referred to Tr-dUrd) of the title uracil derivative, m.p. 125—126°C. For $C_{16}H_{16}N_2O_6$ (332·2) calculated: 57·82% C, 4·85% H, 8·43% N; found: 57·73% C, 5·45% H, 8·29% N.

1-(2-Deoxy-β-D-xylofuranosyl)uracil

A solution of 1-(3-O-benzoyl-2-deoxy-β-D-xylofuranosyl)uracil (0·5 g; 1·5 mmol) in 0·1M methanolic sodium methoxide (50 ml) was kept at room temperature overnight, neutralised with dry Dowex 50 (H^+ form) ion exchange resin, filtered, the resin washed with methanol (50 ml), the filtrate and washings combined, and evaporated. The residue was diluted with water (100 ml), extracted with three 25 ml portions of ether, and the aqueous phase evaporated. The residue was coevaporated with three 50 ml portions of ethanol and then crystallised from ethanol (5 ml), ether being added to the turbidity. Yield, 201 mg (59%), m.p. 160—162°C. For $C_9H_{12}N_2O_5$ (228·2) calculated: 47·36% C, 5·30% H, 12·27% N; found: 47·42% C, 5·32% H, 12·31% N. NMR spectrum (hexadeuteriodimethyl sulfoxide): 6·03 (d, 1 H, $J_{1',2'} = 2·0$, $J_{1',2''} = 8·0$) $H_{1'}$; 1·88 (dd, 1 H, $J_{2',1'} = 2·0$, $J_{gem} = 14·5$) $H_{2'}$; 1·49 (m, 1 H, $J_{2'',1'} = 8·0$, $J_{2'',3'} = 5·0$) $H_{2''}$; 4·34 (m, 1 H, $J_{3',2''} = 5·0$, $J_{3',4'} = 2·5$) $H_{3'}$; 3·50—3·90 (complex m, 3 H) $H_{4'}$ 2 $H_{5'}$; 5·13 (d, 1 H) 3'-OH; 4·58 (t, 1 H) 5'-OH; 5·54 (d, 1 H, $J_{5,6} = 8·1$) H_5; 7·92 (d, 1 H) H_6.

5-(Alkoxymethyl)-2'-deoxyuridines

A mixture of 5-(ethoxymethyl)-2'-deoxyuridine[25] (1 mmol) and dilute (1 : 10) hydrochloric acid (20 ml) was heated at 80°C for 3 h, neutralised by the addition of Dowex 1 × 2 (HCO_3^- form) ion exchange resin, filtered, the filtrate evaporated, and the residue dried. The residue (chromatographically homogeneous 5-hydroxymethyl-2'-deoxyuridine) was refluxed for 3 h in a mixture of the appropriate alcohol (50 ml) and conc. hydrochloric acid (0·5 ml). The mixture was neutralised with triethylamine and chromatographed on one layer of loose silica gel in chloroform–ethanol (9 : 1). The band of the product (R_F 0·35; dUrd, R_F 0·20) was eluted with ethyl acetate (200 ml), evaporated, and dried. The residue, chromatographically homogeneous 5-(methoxymethyl)-2'-deoxyuridine and 5-(1-propyloxymethyl)-2'-deoxyuridine (yields, 75—80%), was directly used in the phosphorylation.

N^4-Dimethyl-2'-deoxycytidine and N^4-(2-Hydroxyethyl)-2'-deoxycytidine

A mixture of 3',5'-di-O-benzoyl-2'-deoxy-4-thiouridine[25] (2·5 mmol) and an ethanolic solution (60 ml) of dimethylamine (20%) or 2-aminoethanol (10%) was heated in an ampoule at 100°C for 8 h, cooled down, evaporated to dryness, the residue diluted with water (100 ml), washed with three 50 ml portions of ether, and the aqueous phase evaporated *in vacuo*. The residue was acidified to pH 3 with hydrochloric acid, filtered through Celite, and the filtrate applied to a column of Dowex 50 (H^+ form) ion exchange resin (100 ml). The column was eluted (Uvicord apparatus) with water to the loss of UV-absorption and conductivity of the eluate, and then with dilute (1 : 10)

aqueous ammonia. The UV-absorbing fraction of the ammonia eluate was evaporated and the content of the residue determined spectrophotometrically at pH 2. Yields: 58% of the crude, chromatographically homogeneous N^4-dimethyl-2′-deoxycytidine (S_1: R_F 0·71; dCyd, R_F 0·63), or 54% of N^4-(2-hydroxyethyl)-2′-deoxycytidine (S_1: R_F 0·68). UV spectrum at pH 2 (both substances): λ_{max} 284 nm, λ_{min} 245 nm. Both crude residues were dried and used in the phosphorylation.

Preparation of Nucleoside 5′-Phosphates

Method A. Phosphorus oxychloride (100 µl) was added under stirring and cooling with ice-cold water to a mixture of the appropriate nucleoside (0·5 mmol) and triethyl phosphate (2·5 ml), the mixture stirred at 0°C for 4 h, diluted with 2M triethylammonium hydrogen carbonate (5 ml), kept at room temperature overnight, evaporated, and the residue chromatographed on 3 sheets of paper Whatman No 3 MM in the system S_1. The mononucleotide bands were eluted with dilute (1 : 100) aqueous ammonia (25 ml), the content of the eluate determined spectrophotometrically at pH 2, and the eluate freeze-dried to afford the following compounds (R_F in S_1 and % yield given): *X* (0·19; 18%), *XI* (0·21; 45%), *XIII* (0·24; 56%), *XIV* (0·27; 58%), *XV* (0·22; 27%), *XVIII* (0·22; 50%), *XIX* (0·29· 45%), *XXIII* (0·20; 52%), *XXXIII* (0·18; 30%), *XXXIV* (0·16; 28%), *XL* (0·16; 29%), *XLIV* (0·29, 60%), *XLV* (0·30, 52%), *XLVI* (0·33; 32%), *XLVII* (0·28, 54%). Compound *XVI* (R_F 0·24 in S_1) was prepared from 1-(3-O-benzoyl-2-deoxy-β-D-xylofuranosyl)uracil (see above) by a similar procedure but the treatment with the buffer solution was followed by heating with 0·1M sodium hydroxide in 50% aqueous methanol for 2 h at 50°C; yield, 56%. Compound *XLIII* (R_F 0·12 in S_1) was prepared by an additional heating of compound *XLIV* in dilute (1 : 10) hydrochloric acid (2 ml per 0·2 mmol of *XLIV*) for 2 h at 80°C and rechromatography in the solvent system S_1; yield, 85%.

Method B. The incubation mixture (100 µl) contained 5 µmol of the nucleoside, 50 µmol of phenyl phosphate sodium salt, 50 µmol of sodium acetate (resulting pH 5·5), and 30 e.u. of the carrot phosphotransferase[10]; incubated at 37°C for 5 h. The mixture was separated by chromatography in the system S_1, the product eluted with dilute (1 : 100) aqueous ammonia, and the eluate freeze-dried to afford the following compounds (% yield given): *XVII* (22%), *XX* (19%), *XXI* (16%), and *XXII* (17·5%).

2′-Deoxyuridine 3′,5′-Diphosphate (*XXVIII*)

Compound *XXVIII* was prepared according to method *A* using 200% of phosphorus oxychloride (6 h at room temperature) in 40% yield (R_F 0·02 in S_1) along with 42% of dUMP ammonium salt (R_F 0·15 in S_1), identical with an authentic material.

1-(β-D-Arabinofuranosyl)uracil 3′-Phosphate (*XXV*)

A solution containing 1-(β-D-arabinofuranosyl)cytosine 3′-phosphate[43,44] ammonium salt (0·35 g; 1 mmol), sodium hydrogen sulfite (2 g), and water (8 ml) was kept at room temperature overnight, adjusted to pH 9 with saturated aqueous barium hydroxide (about 50 ml), heated to 80°C, filtered while hot through Celite, washed with hot water (50 ml), the filtrate and washings combined, cooled down, and adjusted to pH 3 by the addition of Dowex 50 (H^+ form) ion exchange resin. The resin was then filtered off, the filtrate concentrated to the volume of about 50 ml, the concentrate (pH 6·5—7·0) filtered through Celite, the filtrate evaporated, and the residue chromatographed on 4 sheets of paper Whatman No 3 MM in the system S_1. Bands of the product (R_F 0·12) were

eluted with dilute (1 : 100) aqueous ammonia (50 ml), the eluate evaporated, the residue dissolved in a small amount of water, and freeze-dried. Yield, spectrophotometrically: 49%. UV spectrum (pH 2): λ_{max} 261 nm.

2′-Deoxyuridine 5′-Phosphite (*I*)

A mixture of 3′-O-benzoyl-2′-deoxyuridine (332 mg; 1 mmol), dimethylformamide (3 ml), triphenyl phosphite (1·5 ml), and 6M hydrogen chloride in dimethylformamide (0·5 ml) was stirred to obtain a solution which was then kept at room temperature for 3 days, treated with 50% aqueous methanol (100 ml) and triethylamine (10 ml), the mixture heated at 50°C for 2 h, and concentrated to half of the original volume. The concentrate was washed with three 20 ml portions of ether, the aqueous phase evaporated, and the residue chromatographed on 4 sheets in the system S_1 to afford 61% of the chromatographically homogeneous compound *I*, R_F 0·45 (in S_1).

2′-Deoxyuridine 5′-Methanephosphonate (*II*)

3′-O-Benzoyl-2′-deoxyuridine (1 mmol) and N,N′-dicyclohexylcarbodiimide (2·0 g) were added to the pyridine solution (6 ml) of methanephosphonic acid pyridinium salt (3 mmol), the mixture kept at room temperature for 5 days, diluted with water (1 ml), kept for 1 h, and processed analogously to compound *I*. Yield, 24·5% of the ammonium salt of compound *II*, R_F 0·42 (in S_1)

2′-Deoxyuridine 5′-Hydroxymethanephosphonate (*III*)

To a solution of hydroxymethanephosphonic acid (2 mmol) in dimethylformamide (5 ml) there was added 0·65 g (4 mmol) of N,N′-carbonyldiimidazole, the mixture stirred at room temperature for 2 h, treated with 2′-deoxyuridine (228 mg; 1 mmol), the whole stirred under exclusion of atmospheric moisture for 3 days, diluted with water (20 ml), and evaporated. The residue was chromatographed on 4 sheets of paper Whatman No 3 MM in the system S_1. The bands were processed as usual to afford 65% of the chromatographically homogeneous ammonium salt of compound *III*, R_F 0·42 (in S_1).

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