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## Application of chromatographic retention data in an investigation of a quantitative structure–nucleotide incorporation rate relationship

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### SUMMARY

A series of 5-alkyl-, 5-alkenyl- and 5-alkynyl-substituted deoxyuridines and their triphosphate derivatives were synthesized and studied in DNA polymerase reactions. The initial rate of incorporation of the derivatives catalysed by Klenow fragment DNA polymerase enzyme (*E. coli*) was measured. Calf thymus DNA and synthetic poly (dA–dT) served as templates. The rate values were expressed as a percentage relative to the incorporation rate of natural substrate dTTP.

The high-performance liquid chromatographic (HPLC) retention behaviours of the nucleoside derivatives were investigated on silica and reversed-phase stationary phases using various mixtures of ethyl acetate–methanol and methanol–water, as respectively, mobile phases. According to the results of principal component analysis, the HPLC retention data describe the hydrophobic properties of the compounds. The inclusion complex stability constants of the derivatives with cyclodextrins determined by reversed-phase thin-layer chromatography served as a measure of the steric properties of the substituents. The electronic properties of the 5-substituents were characterized by the Swain–Lupton inductive and resonance parameters.

The results of the stepwise linear regression analysis of the nucleotide incorporation rate data and the above-mentioned physico-chemical data revealed the importance of the electronic, steric and hydrophobic properties of the substituents in the DNA polymerase reactions. The importance of the steric parameter was more significant when the poly (dA–dT) template was used instead of the random base sequence template (calf thymus DNA).

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### INTRODUCTION

Nucleotides as the building blocks of the DNA chain play an essential role in life-functions. The possible incorporation of nucleotide analogues into DNA can have crucial effects on its capacity to transmit genetic information. A great number of nucleoside derivatives have already been synthesized and tested as potential antiviral

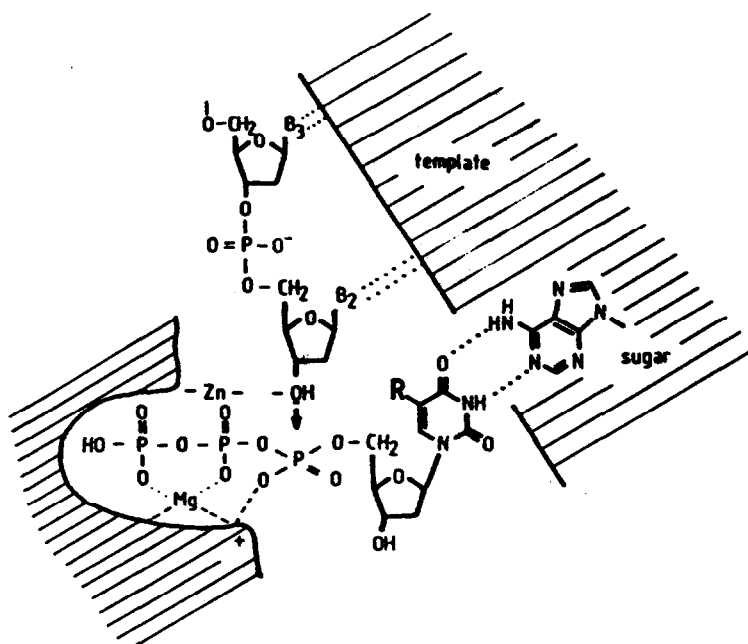


Fig. 1. Model of the nucleotide incorporation reaction.

and antitumour agents, as reviewed by De Clercq<sup>1</sup>. Their activity can be related to their ability to incorporate into DNA or to influence the biosynthesis of DNA.

A series of 5-substituted 2'-deoxyridine-5'-triphosphates have synthesized and their initial incorporation rates into DNA have been measured. A non-linear relationship between the chain length of the 5-substituents and the incorporation rate was observed<sup>2-4</sup>. The effect of chain branching was more significant when the synthetic poly(dA-dT) template was used<sup>4</sup>.

To reveal the molecular parameters involved in DNA replication reactions catalysed by DNA polymerase enzyme (Fig. 1) the physico-chemical properties (hydrophobic, steric and electronic parameters according to Hansch<sup>5</sup>) of the corresponding nucleosides were characterized by their chromatographic retention data.

Reversed-phase high-performance liquid chromatographic (HPLC) retention data were used as a measure of hydrophobicity<sup>6</sup>; retention data on silica stationary phase have already been used as a measure of the adsorption properties<sup>7</sup> of triazine derivatives. The importance of the steric parameters in the nucleotide incorporation reactions was established in a previous study<sup>8</sup>. The molar refractivities (*MR*) are not sensitive to the chain branching of the substituents, although they may influence significantly the incorporation rate. We assumed that cyclodextrin inclusion complex stability data can give information not only about the volume but also the shape of the substituents, and therefore the possible use of complex stability constants was also investigated in this study.

Principal component analysis and stepwise regression analysis were applied for selecting the most important physico-chemical properties of the derivatives that play an important role in DNA replication reactions.

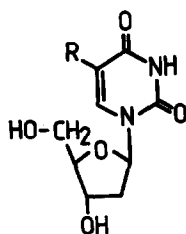


Fig. 2. Structures of the deoxyuridine derivatives investigated.

## EXPERIMENTAL

The structures of the investigated compounds are shown in Fig. 2. The syntheses of the compounds have been described elsewhere<sup>2-4,9</sup>. The compounds were chromatographically pure. Conditions for the measurement of the relative initial incorporation rates of the derivatives into calf thymus DNA (RATE%) and poly (dA-dT) (dAdT%) have already been published<sup>2-4</sup>. The structures of the derivatives investigated in this study, their incorporation rate data (RATE and dAdT%) and their physico-chemical parameters obtained from the Hansch-Leo compilation<sup>10</sup> are listed in Table I. The hydrophobic substituent constant ( $\pi$ ) defined by Hansch and

TABLE I

INVESTIGATED NUCLEOTIDE DERIVATIVES, THEIR INITIAL INCORPORATION RATES INTO CALF THYMUS DNA (RATE%) AND INTO POLY(dA-dT) (dAdT%) AND THEIR PHYSICO-CHEMICAL PARAMETERS OBTAINED FROM THE LITERATURE<sup>2-4,10</sup>

No.	R	RATE%	dAdT%	$\pi$	MR	$\mathcal{F}^a$	$\mathcal{R}^a$
1	H	41	97	0.00	1.03	0.00	0.00
2	Ethyl	18	59	1.02	10.30	0.00	-0.10
3	Isopropyl	6	1	1.53	14.96	-0.05	-0.10
4	sec.-Butyl	8	21	2.07	19.61	-0.06	-0.12
5	tert.-Butyl	6	3	1.98	19.63	-0.07	-0.13
6	Pentyl	6	19	2.67	24.26	-0.06 <sup>b</sup>	-0.08 <sup>b</sup>
7	Hexyl	4	2	3.21	28.91	-0.06 <sup>b</sup>	-0.08 <sup>b</sup>
8	Vinyl	89	96	0.82	10.99	0.07	-0.08
9	(E)-Butynyl	68	80	1.90	20.29	0.03	-0.08
10	(E)-Pentenyl	37	50	2.44	24.94	0.03 <sup>b</sup>	-0.08 <sup>b</sup>
11	(E)-Hexenyl	26	43	2.99	29.59	0.03 <sup>b</sup>	-0.08 <sup>b</sup>
12	(E)-Heptenyl	19	2	3.53	34.24	0.03 <sup>b</sup>	-0.08 <sup>b</sup>
13	(E)-Octenyl	12	0.2	4.07	38.89	0.03 <sup>b</sup>	-0.08 <sup>b</sup>
14	Propynyl	99	69	0.94	14.20	0.15	-0.08
15	Butynyl	74	65	1.48	18.85	0.15 <sup>b</sup>	-0.08 <sup>b</sup>
16	Hexynyl	26	53	2.56	28.15	0.15 <sup>b</sup>	-0.08 <sup>b</sup>
17	Heptynyl	21	52	3.10	32.80	0.15 <sup>b</sup>	-0.08 <sup>b</sup>
18	Octynyl	13	8	3.64	37.45	0.15 <sup>b</sup>	-0.08 <sup>b</sup>

<sup>a</sup> $\mathcal{F}$  and  $\mathcal{R}$  are the Swain-Lupton type<sup>10</sup> inductive and resonance effects, respectively.

<sup>b</sup>Estimated data from values of propyl, propenyl, propynyl.

Leo<sup>10</sup> and the molar refractivities of the 5-substituents with longer chain lengths were calculated on the basis of the additivity rule<sup>10</sup>.

The HPLC measurements were carried out with a Liquopump M312 pump, a variable-wavelength UV detector (Labor-MIM, Budapest, Hungary) and a Rheodyne (Cotati, CA, U.S.A.) injector (20- $\mu$ l loop). Retention time measurements were made with a Waters 740 Data Module (Millipore-Waters, Milford, MA, U.S.A.). Compounds were dissolved in methanol at a 0.1 mg/ml concentration.

#### Reversed-phase chromatographic conditions

An RP-8 (5- $\mu$ m) column (150  $\times$  4.6 mm I.D.) (Perkin-Elmer, Norwalk, CT, U.S.A.) was used with methanol-water mixtures as the mobile phase. The methanol concentration ranged from 40 to 60% (v/v) in 5% steps. A flow-rate of 1.00 ml/min was applied. Peaks were detected at 260 nm. The dead time was determined by injection of 1% sodium nitrate solution.

#### Normal-phase chromatographic conditions

LiChrosorb Si 60 (5 $\mu$ m) (Merck, Darmstadt, F.R.G.) material was packed into a 250  $\times$  4.6 mm I.D stainless-steel column by Bioseparation Techniques (Budapest, Hungary). The mobile phase composition ranged from 10 to 25% (v/v) methanol in ethyl acetate in 5% steps. Detection was effected at 260 nm.

The logarithmic values of the capacity factors ( $\log k'$ ) of the compounds mea-

TABLE II

INTERCEPT AND SLOPE VALUES OBTAINED BY HPLC SILICA (*SII*, *SIS*) AND REVERSED-PHASE (*RPI*, *RPS*) METHODS, THE CORRESPONDING CORRELATION COEFFICIENTS, *r*, THE INCLUSION COMPLEX STABILITY CONSTANTS DETERMINED BY RP-TLC (*K*) AND THE FIRST PRINCIPAL COMPONENTS OBTAINED FROM THE CHROMATOGRAPHIC RETENTION DATA (*PCI*) FOR THE INVESTIGATED NUCLEOSIDE DERIVATIVES.

<i>R</i>	<i>SII</i>	<i>SIS</i> $\times 10^{-2}$	<i>r</i>	<i>RPI</i>	<i>RPS</i> $\times 10^{-2}$	<i>r</i>	<i>K</i>	<i>PCI</i>
H	0.329	-2.37	0.99	0.424	-2.65	0.99	0	2.36
Ethyl	0.246	-3.35	0.99	0.590	-2.39	0.96	0	2.85
Isopropyl	0.015	-2.32	0.99	0.724	-2.39	0.98	0	1.70
<i>sec.</i> -Butyl	-0.416	-0.89	0.99	1.299	-2.82	0.98	0	-0.51
<i>tert.</i> -Butyl	-0.372	-1.12	0.99	1.301	-2.80	0.99	1.03 $\pm$ 0.31	-0.27
Pentyl	-0.459	-0.88	0.99	2.041	-3.53	0.99	2.74 $\pm$ 0.25	-0.52
Hexyl	-0.439	-1.20	0.99	3.001	-4.90	0.99	3.12 $\pm$ 0.24	-2.24
Vinyl	-0.013	-2.52	0.99	0.613	-2.44	0.99	1.40 $\pm$ 0.27	1.81
( <i>E</i> )-Butenyl	-0.394	-1.23	0.99	0.755	-1.56	0.99	1.87 $\pm$ 0.28	0.64
( <i>E</i> )-Pentenyl	-0.405	-1.47	0.99	1.676	-2.87	0.99	0.67 $\pm$ 0.11	-0.35
( <i>E</i> )-Hexenyl	-0.417	-1.46	0.99	2.224	-3.30	0.99	1.83 $\pm$ 0.31	-0.87
( <i>E</i> )-Heptenyl	-0.414	-1.45	0.99	2.953	-4.09	0.99	3.40 $\pm$ 0.26	-1.62
( <i>E</i> )-Octenyl	-0.349	-1.85	0.99	3.434	-4.45	0.99	4.60 $\pm$ 0.24	-1.67
Propynyl	-0.042	-2.31	0.99	0.699	-2.69	0.98	0.27 $\pm$ 0.07	1.61
Butynyl	0.063	-3.15	0.99	0.704	-2.15	0.99	0.66 $\pm$ 0.18	2.43
Hexynyl	-0.453	-1.09	0.99	2.287	-4.02	0.99	0.70 $\pm$ 0.14	-1.55
Heptynyl	-0.399	-1.54	0.99	2.297	-4.70	0.99	1.60 $\pm$ 0.29	-1.84
Octynyl	-0.393	-1.57	0.99	3.268	-4.72	0.99	2.77 $\pm$ 0.19	-1.98

sured in reversed-phase chromatographic systems were plotted against the methanol concentrations in the mobile phase. Similar plots were constructed for the log  $k'$  values obtained in the normal chromatographic mode against methanol concentration. For each compound a straight line could be fitted to at least four data points. The correlation coefficients were always higher than 0.97. The slope and the intercept values for the straight lines obtained in both the reversed-phase (*RPS*, *RPI*) and normal-phase modes (*SIS*, *SII*) were calculated and are given in Table II.

#### *Inclusion complex stability measurements*

Reversed-phase thin-layer chromatography (TLC) was used to study the inclusion complexes formed by the nucleoside derivatives with  $\beta$ -cyclodextrins. The determinations of the stability constants are based on the difference in lipophilicity between the complexed and free forms of the compounds<sup>11</sup>.

Silufol UV254 plates (Kavalier, Sklárny, Czechoslovakia) were impregnated with paraffin oil as described elsewhere<sup>12</sup>. The nucleoside derivatives were dissolved in methanol at a concentration of 3 mg/ml; 2  $\mu$ l of each solution were spotted on the plates separately. The mobile phase contained ethanol in the concentration range 0–15% (v/v) in steps of 5%, as it is miscible with water and forms only a weak inclusion complex with  $\beta$ -cyclodextrin<sup>13,14</sup>.  $\beta$ -Cyclodextrin was obtained from Chinoïn (Budapest, Hungary) and was added to the eluent at the concentration of 15 mM. Cyclodextrin-free eluents served as controls. After chromatographic development, the plates were dried at 105°C and the nucleoside spots were detected under UV light. For each experiment four replicate determinations were carried out. The inclusion complex stability constants ( $K$ ) were calculated as described elsewhere<sup>15</sup> and are given in Table II.

Principal component analysis<sup>16</sup> was applied in order to find similarities and dissimilarities between the biological, physico-chemical and chromatographic parameters, taking into consideration all compounds simultaneously. For a better understanding of the results of the principal component analysis, the non-linear map of the principal component loadings and variables was also calculated<sup>17</sup>. The calculation was run to 99.9% variance explained (*i.e.*, as many principal components were calculated as needed to explain more than 99.9% of the inherent variance of the original data matrix). Stepwise linear regression analyses of the data obtained were carried out on a IBM AT compatible personal computer using Labsware program package (Compudrug, Budapest, Hungary).

## RESULTS AND DISCUSSION

The inclusion complex stability constants of the derivatives show an interesting dependence on the 5-substituents, *i.e.*, the longer the chain length, the higher is the complex stability. Double and triple bonds in the side-chain decrease the inclusion complex stability constants. As the inner side of the cyclodextrin ring is non-polar, it can be assumed that the side-chain of the derivatives (they are also non-polar) protrudes into the ring as shown schematically in Fig. 3.

Principal component analysis was carried out using the variables listed in Table I and Table II. The eighteen deoxyuridine derivatives were used as the observations. The non-linear map of the variables obtained is shown in Fig. 4. The variables describ-

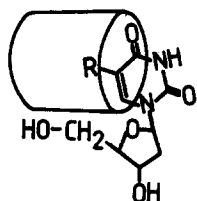


Fig. 3. Possible inclusion complex formation of the deoxynucleosides with  $\beta$ -cyclodextrin.

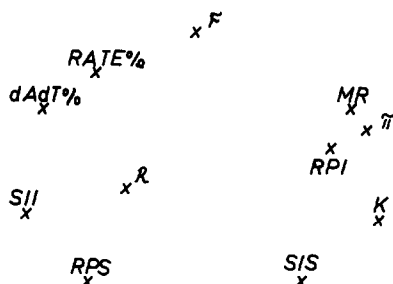


Fig. 4. Non-linear map of the principal component loadings of the incorporation rate, physico-chemical, chromatographic retention and inclusion complex stability parameters. Number of iterations, 74; maximum error, 0.031.

ing similar properties of the compounds are near to each other and the variables describing dissimilar properties are widely separated on the non-linear map of principal component loadings. As it can be seen in Fig. 4, the incorporation rate data are close to each other, and the molar refractivity ( $MR$ ),  $\pi$  and  $RPI$  are close to each other as a measure of the hydrophobicity of the compounds<sup>8</sup>. The electronic parameters ( $\mathcal{F}$ ,  $\mathcal{R}$ ), silica retention parameters and reversed-phase slope ( $RPS$ ) parameter form distinct variables. The inclusion complex stability constant ( $K$ ) is relatively close to the hydrophobic parameters. The non-linear map of the compounds is shown in Fig. 5. The compounds having alkyl, alkenyl and alkynyl substituents are situated in one direction, and compounds with longer chain lengths are situated in a diagonal direction.

Another principal component analysis was carried out using only the four HPLC retention parameters ( $RPI$ ,  $RPS$ ,  $SII$  and  $SIS$ ). Fig. 6 shows the non-linear map so obtained of the principal component loadings. When there is a double or triple bond in the 5-substituent, the nucleosides form a group with the compound having an alkyl substituent with one more  $CH_2$  group, as can be observed in Fig. 6. The first principal component (PC1) of the chromatographic parameters describes the 74% variance of the four variables, and together with the second one more than 93%

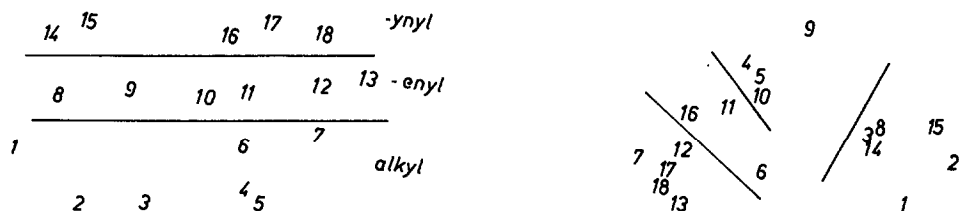


Fig. 5. Non-linear map of the principal components of the compounds of all the variables. The numbers represent the compounds listed in Table I. Number of iterations, 80; maximum error, 0.020.

Fig. 6. Non-linear map of the principal components taking into consideration only the HPLC retention parameters ( $SII$ ,  $SIS$ ,  $RPI$ ,  $RPS$ ). The numbers represent the compounds listed in Table I. Number of iterations, 98; maximum error, 0.0057.

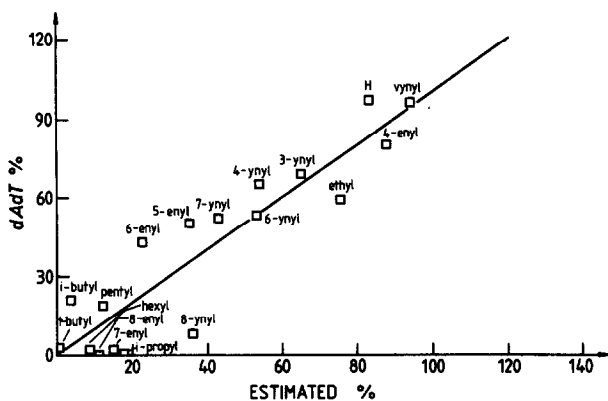


Fig. 7. Plot of the measured and estimated dAdT% values according to eqn. 1.

of the variance is explained. The PC1 variable was also used as a possible independent variable in the stepwise linear regression analysis.

In order to reveal the most important properties of the compounds which are involved in the nucleotide incorporation rate, stepwise regression analysis was carried out using the RATE% and dAdT% parameters as dependent variables. The possible independent variables were the  $\pi$ ,  $MR$ ,  $\mathcal{F}$ ,  $\mathcal{R}$ ,  $SII$ ,  $SIS$ ,  $RPI$ ,  $RPS$ ,  $K$ ,  $\log K$ , PC1 variables. As was found previously<sup>8</sup>, the electronic ( $\mathcal{F}$ ), steric ( $MR$ ) and hydrophobic ( $RPS$  and  $RPI$ ) parameters are all important.

The best correlation regarding dAdT% as the dependent variable is described by

$$\text{dAdT}\% = 154(\pm 41) \mathcal{F} - 28.8(\pm 3.6)\log K + 25.7 \quad (1)$$

$$n = 18; r = 0.92; s = 14.4; F = 39.2$$

where  $n$  = number of compounds,  $r$  = multiple correlation coefficient,  $s$  = standard error of the the estimate and  $F$  = the Fischer-test value. Eqn. 1 means that the higher

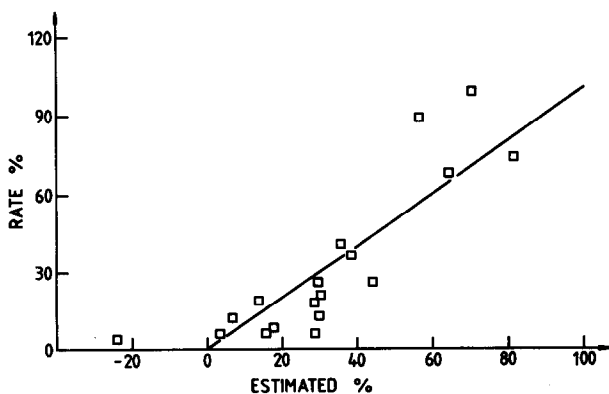


Fig. 8. Plot of the measured and estimated RATE% values according to eqn. 2.

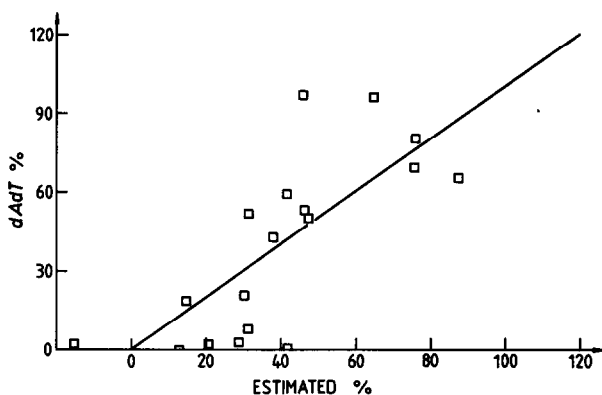


Fig. 9. Plot of the measured and estimated dAdT% values according to the equation  $dAdT\% = 2189 (\pm 589) RPS + 203 (\pm 70) \mathcal{F} + 104$  ( $r=0.75$ ;  $s=24$ ;  $F=9.3$ ).

is the inductive effect of the 5-substituent and the smaller is the stability of the inclusion complex with cyclodextrin, the higher is the percentage incorporation rate measured with poly(dA-dT) as template. A plot of the measured and estimated dAdT% values by eqn. 1 is shown in Fig. 7.

When the random sequence template (calf thymus DNA) (RATE%) is considered, the best correlation using only two independent variables is described by

$$RATE\% = 2006 (\pm 417) RPS + 241 (\pm 50) \mathcal{F} + 88.7 \quad (2)$$

$$n = 18; r = 0.85; s = 17.0; F = 19.5$$

A plot of the measured and estimated RATE% values according to eqn. 2 is shown in Fig. 8. The *RPS* variable reveals the sensitivity of the retention change caused by increasing methanol concentration and it can be regarded as a measure of the contact

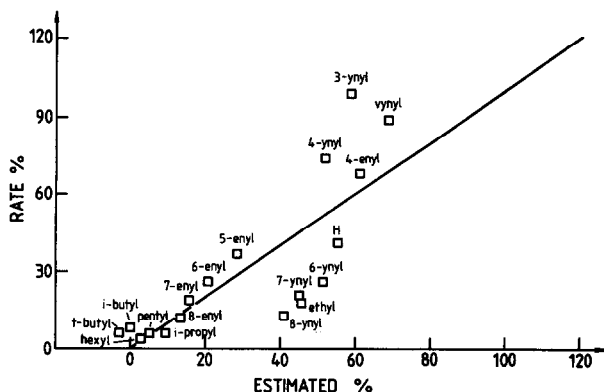


Fig. 10. Plot of the measured and estimated RATE% values according to the equation  $RATE\% = 196 (\pm 56) \mathcal{F} - 18 (\pm 5) \log K + 19$  ( $r=0.79$ ;  $s=20$ ;  $F=13$ ).



TABLE III  
CORRELATION MATRIX OF VARIABLES LISTED IN TABLES I AND II

	RATE%	dAdT%	$\pi$	MR	$\mathcal{F}$	SII	SIS	RPI	RPS	Log K	PCI
RATE%	1.00	0.78	-0.56	-0.44	0.54	0.44	-0.46	-0.55	0.54	-0.58	0.56
dAdT%	0.78	1.00	-0.71	-0.63	0.38	0.55	-0.46	-0.63	0.55	-0.83	0.62
$\pi$	-0.56	-0.71	1.00	0.98	0.09	-0.82	0.58	0.95	-0.79	0.76	-0.91
MR	-0.44	-0.63	0.98	1.00	0.26	-0.81	0.53	0.93	-0.78	0.75	-0.89
$\mathcal{F}$	0.54	0.38	0.09	0.26	1.00	0.02	-0.20	0.17	-0.19	0.00	-0.08
SII	0.44	0.55	-0.82	-0.81	0.02	1.00	-0.89	0.55	-0.57	-0.66	0.89
SIS	-0.46	-0.46	0.58	0.53	-0.20	-0.89	1.00	0.53	-0.43	0.53	-0.79
RPI	-0.55	-0.63	0.95	0.93	0.17	0.55	0.53	1.00	-0.94	0.71	-0.93
RPS	0.54	0.55	-0.79	-0.78	-0.19	-0.57	-0.43	-0.94	1.00	-0.64	0.85
Log K	-0.58	-0.83	0.76	0.75	0.00	-0.66	0.53	0.71	-0.64	1.00	-0.73
PCI	0.56	0.62	-0.91	-0.89	-0.08	0.89	-0.79	-0.93	0.85	-0.73	1.00

hydrophobic surface area<sup>18</sup> of the compounds. The slight difference between the two equations reveals the greater significance of the steric parameter when the template has a strictly alternating base sequence.

When the independent variables are exchanged in the two equations they are also significant in the correlations, but the mathematical statistical characteristics of the equations obtained are worse. The multiple correlation coefficient of eqn. 1 when RPS and  $\mathcal{F}$  are the independent variables is 0.75, and the standard error of the estimate is increased to 24. A plot of the measured and estimated dAdT% values is shown in Fig. 9.

Using  $\log K$  and  $\mathcal{F}$  as independent variables in eqn. 2 resulted in a multiple correlation coefficient of 0.79 and a standard error of the estimate of 20. A plot of the measured and estimated RATE% values shown in Fig. 10 and indicates that five compounds (deoxyuridine and ethyl-, hexynyl-, heptynyl- and octynyldeoxyuridine) form another straight line. For these compounds the estimated RATE% values are higher than the measured values.

The correlation coefficients between each of the variables used in the linear regression analysis are summarized in Table III. It is noticeable that  $\pi$ ,  $MR$ ,  $RPI$  and  $PC1$  parameters show a high intercorrelation as all of them are proportional to the hydrophobicity of the molecules.

We conclude from our calculations that the physico-chemical properties of nucleoside derivatives measured by various chromatographic methods serve as a valuable aid in revealing the most important parameters which are involved in the nucleotide incorporation reactions in the Klenow DNA polymerase system. To obtain a high rate of incorporation of a nucleotide analogue into a DNA, the 5-substituent of the deoxynucleosides should have a high electron-withdrawing character and small hydrophobic surface area and should be sterically small.

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