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PHYSICO-CHEMICAL CHARACTERIZATION OF CYCLIC NUCLEOTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. QUANTITATIVE DETERMINATION OF HYDROPHOBICITY

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SUMMARY

Retention data for 42 adenosine 3′,5′-monophosphate derivatives were measured in a reversed-phase high-performance liquid chromatographic (RPLC) system. Using methanol–water and methanol–phosphate buffer as the mobile phase, the relationship between the volume fraction of organic modifier, φ, and the logarithm of the capacity factor, log k', can be adequately described by a linear regression equation. The resulting log k_w values, obtained by extrapolation of retention to $\varphi = 0$, are correlated with other physico-chemical solute properties that are commonly used to describe quantitatively the hydrophobic nature of solutes.

The results indicate that the retention behaviour of complex molecular structures, including ionic, hydrogen bonding and hydrophobic functions, is only moderately described by the standard Hansch π substituent constant, although the underlying distribution mechanism of both RPLC and a true liquid–liquid system is the solvophobic effect. This is additionally demonstrated by the strong dependence of log k_w on the molecular volume of the substituents.

It is concluded that, rather than attempt to establish correlations between RPLC retention data and distribution coefficients, log k_w values can be used directly as descriptors of a solute's hydrophobicity and may therefore be used in studies of quantitative structure–activity relationships for cyclic nucleotides.

INTRODUCTION

Analogues of adenosine 3′,5′-monophosphate (cAMP) have been systematically used to elucidate the molecular interactions by which cAMP is bound to its receptor proteins[1]. The base moiety of cAMP offers several possibilities for such specific interactions: the N^6 amino-nitrogen may serve as a hydrogen bond donor, and N^1, N^3 and N^7 may act as hydrogen bond acceptors[2]. The purine ring as a whole probably binds to a hydrophobic cleft at the receptor area of, *e.g.*, the regulatory subunit of protein kinase type I[3]. Therefore, it is important to know the contribution

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of a newly introduced substituent to the overall hydrophobicity of cyclic nucleotide derivatives.

Hansch and co-workers[4,5] developed the *n*-octanol–water distribution system as an appropriate model for the determination of the hydrophobic nature of a compound. Since then, solute hydrophobicity has routinely been expressed as its distribution coefficient (log *P*) in the *n*-octanol–aqueous phase system. Log *P* is either determined experimentally by the laborious and time-consuming shaking flask method, or calculated by summing known Hansch π or Rekker's *f* values[6]. Both procedures may not provide reliable figures for the hydrophobicity of cAMP analogues because (i) at any pH, cyclic nucleotides are charged compounds, causing difficulties in log *P* determinations, which are related to the high solubility of water in *n*-octanol and the possible distribution of ion pairs that may be formed by solute ions and buffer components, and (ii) the purine ring contains four nitrogen atoms so that strong electronic interactions between substituents and the heterocyclic base are expected to occur, thereby preventing a simple calculation of log *P* from known hydrophobic substituent constants.

The possibility of using reversed-phase high-performance liquid chromatography (RPLC) for measuring liquid–liquid distribution coefficients of neutral compounds and weak acids and bases has been demonstrated by numerous workers (see refs. 7–12 for extensive references). The results indicate a fundamental correspondence of retention in RPLC and liquid–liquid distribution, so that RPLC retention parameters have been successfully employed either to calculate log *P* values or to describe directly the hydrophobic nature of bioactive compounds in studies on quantitative structure–activity relationships (QSAR)[12–14].

In a previous paper[15], we showed that the negatively charged cyclic nucleotides interact with metal cations in the mobile phase to form nucleotide–metal ion complexes with reduced electronic charge and thus enhanced retention. As a consequence, two different solute species can be used to determine experimentally the effect of substituents on retention, *viz.*, (i) the charged cyclic nucleotide in the absence of cations in the eluent and (ii) the cyclic nucleotide–metal ion complex at saturating cation concentrations with respect to complex formation.

In this work, we determined the retention behaviour of 42 cyclic nucleotides, including base-modified, ribose-modified and phosphate-modified analogues, in the presence and absence of K^+ ions in the mobile phase. From the retention parameters we calculated RPLC group contribution constants, which were subsequently related to other physico-chemical substituent constants. The results provide valuable information about the capability of RPLC for the quantitative determination of the hydrophobic nature of complex solutes containing strongly interacting and charged substituents.

EXPERIMENTAL

A Constametric III (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) liquid chromatograph was used with a Model 7125 sampling valve (Rheodyne, Berkeley, CA, U.S.A.), a Model UV III UV detector (LDC) set at 254 nm and a Servogor Model S recorder (Metrawatt, Nürnberg, F.R.G.). The stainless-steel column (25 cm × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.) was packed by the slurry technique[12]

with 10-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.) known to possess a very high surface coverage of octadecyl ligands[16]. The column was used without further treatment in all experiments. The mobile phase was prepared volumetrically by mixing methanol (Baker HPLC reagent) and doubly distilled water obtained with an all-glass distillation unit (Heraeus-Schott, Mainz, F.R.G.) or potassium phosphate buffer (pH 6.6). When using buffer, the concentration of K^+ ions was adjusted to 100 m*M*.

Samples were dissolved in water and 5 μl of a 0.1 m*M* sample solution was injected into the column. Retention time measurements and the reproducibility of the experimental system have been described previously[15]. Data processing was carried out using a standard computer program for multivariate data analysis.

Fig. 1 shows the structure of adenosine 3',5'-monophosphate and indicates the substituent positions. Table I lists the cyclic nucleotides used in this study and identifies the sources of the compounds.

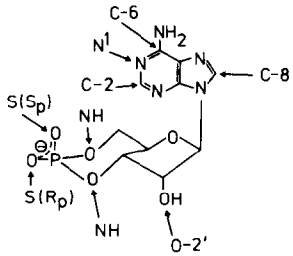

Fig. 1. Structure of adenosine 3',5'-monophosphate and indication of substituent positions.

RESULTS AND DISCUSSION

Retention parameter

Retention in RPLC is described by the solute capacity factor, k', calculated as the normalized solute retention, *viz.*,

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R is the solute retention time and t_0 is the mobile phase hold-up time. A number of studies have shown that the capacity factor (usually in its logarithmic form) is not well suited to describe quantitatively the hydrophobic nature of a solute[7,11,12,20], as compounds with the same k' at a given mobile phase composition do not necessarily exhibit the same retention mechanism. Such differences can be detected by measuring the dependence of log k' on the volume fraction of the organic modifier, φ, in the mobile phase. Plots of log k' *versus* φ for solutes differing in size and/or polarity often reveal large differences with respect to the slope of the resulting curves, giving rise to intersections and reversals of the elution order at certain values of φ. These selectivity differences[12] can be eliminated by using a (hypothetical) solute capacity factor, k_w, for water as the eluent. The k_w values are usually too high to obtain experimentally, and therefore have to be calculated using extrapolation techniques.

TABLE I

NAMES, SUBSTITUENTS, ABBREVIATIONS AND SOURCES OF CYCLIC NUCLEOTIDE ANALOGUES

No.	*Name*	*Source**	*No.*	*Name*	*Source**
1	Adenosine 3′,5′-monophosphate (cAMP)	2		*C-8-substituted:*	
	N-1-substituted:		22	8-Thio-cAMP	4
2	N^1-Oxide-cAMP	1	23	8-Bromo-cAMP	2
3	N^1-Methoxy-cAMP	4	24	8-*p*-Chlorothiophenyl-cAMP (PCTP)	2
	C-2-substituted:		25	8-Thioethyl-cAMP	4
4	2-Methyl-cAMP	4	26	8-Hydroxyisopropyl-cAMP (8-OH-i-P)	5
5	2-Ethyl-cAMP	4	27	8-Methoxy-cAMP	4
6	2-*n*-Propyl-cAMP	4	28	8-Amino-cAMP	4
7	2-*n*-Butyl-cAMP	4	29	8-Methylamino-cAMP (8-MA)	3
8	2-*n*-Hexyl-cAMP	4	30	8-Dimethylamino-cAMP (8-DMA)	4
9	2-Phenyl-cAMP	4	31	8-Hydroxy-cAMP	2
10	2-Chloro-cAMP	4		*Ribose-substituted:*	
11	2-Thiomethyl-cAMP	4	32	2′-O-(2,4-Dinitrophenoxy)-cAMP (DNP)	1
12	2-Thiopropyl-cAMP	4	33	3′-Amino-3′-deoxy-cAMP (3′-NH)	1
	C-6-substituted:		34	5′-Amino-5′-deoxy-cAMP (5′-NH)	1
13	Purine 3′,5′-monophosphate (cPuMP)	1		*Phosphate-substituted:*	
14	Inosine 3′,5′-monophosphate (cIMP)	3	35	Adenosine 3′,5′-monophosphothioate, R_p-isomer [(R_p)-cAMPS]	1
15	6-Chloro-cPuMP	3	36	Adenosine 3′,5′-monophosphothioate, S_p-isomer [(S_p)-cAMPS]	1
16	6-Methylamino-cPuMP (6-MA)	4		*Disubstituted:*	
17	6-Dimethylamino-cPuMP (6-DMA)	2	37	N^6,$O^{2'}$-Di-*n*-butyryl-cAMP (dibutyryl)	2
18	6-Methoxy-cPuMP	1	38	1,N^6-Etheno-cAMP	3
19	6-Benzyloxy-cPuMP	1	39	6-Dimethylamino-cAMPS, R_p-isomer [6-(R_p)-DMA-S]	1
20	6-Thiomethyl-cPuMP	1	40	6-Dimethylamino-cAMPS, S_p-isomer [6-(S_p)-DMA-S]	1
21	6-*n*-Butyrylamino-cPuMP (monobut)	2	41	Guanosine 3′,5′-monophosphate (cGMP)	2
			42	Guanosine 3′,5′-monophosphothioate, S_p-isomer [(S_p)-cGMPS]	1

* 1, Synthesized in our laboratory according to refs. 17–19; 2, Boehringer (Mannheim, F.R.G.); 3, Sigma (Munich, F.R.G.); 4, generous gift from J. P. Miller, SRI International Life Science Division; 5, generous gift from Dr. D. Shugar, Polish Academy of Science.

According to the solubility parameter concept[21], the relationship between solute retention and the composition of binary mobile phases can be described by

$$\log k' = \log k_w + A\varphi^2 - S\varphi \quad (2)$$

where A and S are constants for a given solute–organic modifier combination. It has been shown for model solutes[22] that over limited ranges of binary compositions, a simple linear relationship can be used as a good approximation of eqn. 2, *viz.*,

$$\log k' = \log k_w - S\varphi \quad (3)$$

Fig. 2 shows a plot of log k' *versus* φ_M, the methanol content in a methanol–water eluent, for some cAMP analogues. Under such mobile phase conditions, *i.e.*, in the absence of cations, cyclic nucleotides carry one net negative charge[15] and elute at low values of φ_M, thus enabling a comparison to be made between extrapolated and measured log k_w values. We selected those solutes for which the retention can be conveniently measured at a minimum of six different values of φ_M, including φ_M = 0. The resulting data from regression analysis and measured log k_w values are given in Table II. Note that measured log k_w values were not included in the data set used for extrapolation.

The results in Fig. 2 and Table II indicate that eqn. 2 describes excellently the retention over the volume fraction range studied with multiple correlation coefficients, r, always ≥ 0.999. However, extrapolated log k_w values tend to be slightly lower than those obtained experimentally. In fact, Schoenmakers *et al.*[23] have shown that a correction term had to be included in eqn. 2 to describe satisfactorily the retention in water-rich eluents. This term accounts for changes in composition and polarity of the stationary phase due to limited sorption of the organic modifier at φ values below 0.10. Obviously, the solvation properties of the octadecyl-coated silica

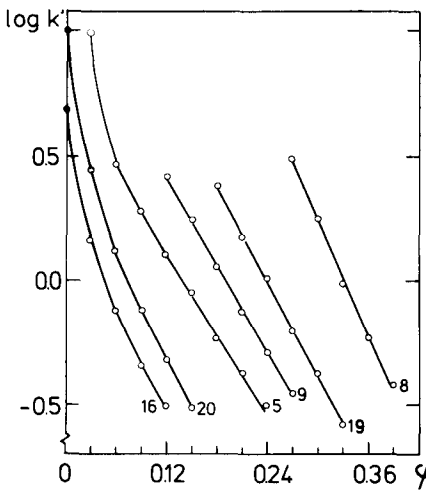

Fig. 2. Plot of log k' versus φ_M, the volume fraction of methanol in the mobile phase, for cyclic nucleotides numbered according to Table I.

TABLE II

CALCULATION OF LOG k_w VALUES FROM THE RELATIONSHIP BETWEEN THE VOLUME FRACTION OF METHANOL, φ_M, AND LOG k' FOR CHARGED CYCLIC NUCLEOTIDES

Regression analysis was performed using the quadratic eqn. 2 and the linear eqn. 3 for the extrapolation of retention to $\varphi_M = 0$. Figures in parentheses are the standard deviations of the regression coefficients of the resulting log k_w values, and r is the (multiple) regression correlation coefficient. Included are measured log k_w values using neat aqueous eluent.

*No.**	*Substituent**	*Regression*				*Measured log k_w*
		Quadratic log k_w	*r*	*Linear log k_w*	*r*	
4	2-Methyl	0.47 (0.01)	0.999	0.40 (0.05)	0.994	0.48
5	2-Ethyl	1.33 (0.04)	0.999	1.14 (0.13)	0.997	1.35
10	2-Chloro	0.60 (0.05)	0.999	0.49 (0.08)	0.997	0.64
11	2-Thiomethyl	0.78 (0.09)	0.999	0.66 (0.10)	0.998	0.86
15	6-Chloro	0.36 (0.02)	0.999	0.29 (0.06)	0.993	0.37
16	6-MA	0.65 (0.05)	0.999	0.52 (0.09)	0.994	0.69
20	6-Thiomethyl	0.99 (0.06)	0.999	0.84 (0.12)	0.998	1.01
21	6-Monobut	1.04 (0.05)	0.999	0.85 (0.15)	0.994	1.06
23	8-Bromo	0.38 (0.02)	0.999	0.30 (0.07)	0.991	0.39
26	8-OH-i-P	0.62 (0.05)	0.999	0.51 (0.08)	0.997	0.67
27	8-Methoxy	0.50 (0.03)	0.999	0.40 (0.07)	0.995	0.52
29	8-MA	0.67 (0.04)	0.999	0.57 (0.07)	0.996	0.70

* Numbering and abbreviations as in Table I.

gel surface are not independent of the organic modifier content in the mobile phase, causing a breakdown of the solubility parameter model at high water contents[23–25].

These results, together with the observation that the curvature of the plot of log k' *versus* φ_M (Fig. 2) is mainly caused by log k' values obtained at very low values of φ (*i.e.*, $\varphi_M \leqslant 0.06$), suggested that exclusion of these data from regression analysis may allow the application of eqn. 3 as a valuable approximation. If linear regressions are restricted to φ_M values as low as 0.06, a good fit of the data to eqn. 3 is observed (Table II). Of greater importance is the fact that extrapolated log k_w values resulting from eqn. 2 [log $k_{w(2)}$] and eqn. 3 [log $k_{w(3)}$] show a strong mutual correlation and, further, that a very reasonable agreement exists between extrapolated and measured [log $k_{w(exp)}$] data, irrespective of the kind of regression analysis. This is demonstrated by eqns. 4–6:

$$\log k_{w(3)} = 0.867\ (0.015) \log k_{w(2)} - 0.026 \quad (4)$$
$$n = 12;\ r = 0.9986;\ F = 3591;\ \text{S.D.} = 0.014$$

$$\log k_{w(exp)} = 1.009\ (0.022) \log k_{w(2)} + 0.023 \quad (5)$$
$$n = 12;\ r = 0.9976;\ F = 2056;\ \text{S.D.} = 0.022$$

$$\log k_{w(exp)} = 1.160\ (0.032) \log k_{w(3)} + 0.055 \quad (6)$$
$$n = 12;\ r = 0.9962;\ F = 1301;\ \text{S.D.} = 0.027$$

where the values in parentheses are the standard deviations of the regression coefficients, and n, r, F and S.D. are the number of data points, the linear regression correlation coefficient, the variance ratio value and the standard deviation of regression, respectively. The slope of eqn. 6 indicates that when using linear extrapolation, a small underestimation of the contribution of a substituent to retention, compared with log $k_{w(exp)}$, has to be taken into consideration.

A number of studies have shown[10–12] that an excellent mutual agreement exists between log $k_{w(3)}$, *i.e.* log k_w obtained by linear extrapolation of retention data measured at φ values between 0.1 and 0.9, and the liquid–liquid distribution coefficient, log P. These findings indicate that a hypothetical RPLC system composed of ODS stationary phase and an aqueous mobile phase in many respects resembles the standard *n*-octanol–water distribution system. This may not be true for the real aqueous RPLC used to measure log k_w because a reduction in the organic modifier content to below 10% causes drastic changes in the structure and solvation properties of the stationary phase[23]. Therefore, we conclude that the simple linear approach is appropriate for the determination of the hydrophobic nature of cyclic nucleotides. This conclusion is further substantiated by the finding that the inclusion of a quadratic term in eqn. 3 neither leads to a precise prediction of log $k_{w(exp)}$ nor improves the correlation between extrapolated and measured values. In addition, the use of quadratic or even more complex[23] functions for purposes of extrapolation requires the determination of a large number of capacity factors per solute, particularly at low values of φ, owing to a greatly increased uncertainty in the intercept value of log k_w on introduction of a φ^2 term, so that practical reasons also point to the use of the linear eqn. 3 for the determination of hydrophobic parameters of cyclic nucleotides[20].

Hydrophobic parameters for cyclic nucleotides

As cyclic nucleotides carry one negative charge at the phosphate moiety in methanol–water eluents, giving rise to strong interactions with the mobile phase, a noticeable retention is observed only for compounds that carry hydrophobic substituents (Table II). We have previously demonstrated[15] that nucleotides effectively bind metal cations in the mobile phase. In this way, the charge of the solute is largely neutralized so that the nucleotide–cation complexes showed enhanced retention. Therefore, we were able to measure the retention data for 42 polar and non-polar cAMP analogues using mobile phases with volume fractions of methanol (φ_M) between 0.15 and 0.51 in 100 m*M* potassium phosphate buffer (pH 6.6) (Table III). As demonstrated in the previous section, the restriction to φ_M values above 0.10 allows the use of eqn. 3 for the appropriate extrapolation of retention data to $\varphi_M = 0$. The upper limit of the accessible volume fraction range is given by the limited solubility of potassium phosphate in methanol-containing solvents. For the compounds studied, an excellent correlation is found between log k' and φ_M (Table IV), with linear correlation coefficients being $\leqslant 0.999$ in only a few instances.

In order to investigate the capability of log k_w (given in Table IV) as a measure of the hydrophobic nature of cyclic nucleotides, retention data were related to other physico-chemical parameters that are commonly used for the same purpose. For a clear discussion it is convenient to introduce an RPLC group contribution constant, τ, which describes the contribution of a substituent to retention. τ may be defined[26] as

$$\tau = \log (k'_j/k'_i) \qquad (7)$$

TABLE III

ISOCRATIC LOG k' VALUES OF cAMP ANALOGUES FOR DIFFERENT VOLUME FRACTIONS OF METHANOL, φ_M, IN 100 mM PHOSPHATE BUFFER (pH 6.6)

*No.**	*Substituent**	*Volume fraction, φ_M*				
		0.51	*0.48*	*0.45*	*0.42*	*0.39*
8	2-*n*-Hexyl	0.666	0.790	0.930	1.068	
19	6-Benzyloxy	0.470	0.593	0.722	0.848	
24	8-PCTP	0.407	0.542	0.684	0.819	
37	Dibutyryl	0.191	0.310	0.435	0.553	
32	2'-DNP	0.123	0.241	0.362	0.479	
12	2-Thiopropyl	0.059	0.171	0.293	0.412	0.553
9	2-Phenyl	0.029	0.137	0.277	0.404	0.543
7	2-*n*-Butyl	0.024	0.126	0.272	0.381	0.543
40	6-(S_p)DMA-S	−0.195	−0.065	0.043	0.171	0.297
6	2-*n*-Propyl		−0.201	−0.110	0.000	0.129
20	6-Thiomethyl		−0.232	−0.130	−0.023	0.075
39	6-(R_p)DMA-S		−0.232	−0.130	−0.023	0.094
17	6-DMA		−0.282	−0.184	−0.082	0.036
21	6-Monobut			−0.258	−0.140	−0.047
11	2-Thiomethyl				−0.286	−0.167
5	2-Ethyl					−0.189
15	6-Chloro					−0.286
10	2-Chloro					−0.286
23	8-Bromo					−0.402
16	6-MA					−0.402
18	6-Methoxy					−0.258
25	8-Thioethyl					−0.431
26	8-OH-i-P					
36	(S_p)cAMPS					
38	1,N^6-Etheno					
27	8-Methoxy					
35	(R_p)cAMPS					
29	8-MA					
1	cAMP					
13	cPuMP					
33	3'-NH-cAMP					
42	(S_p)cGMPS					
34	5'-NH-cAMP					
4	2-Methyl					
30	8-DMA					
28	8-Amino					
22	8-Thio					
3	N^1-Methoxy					
14	cIMP					
41	cGMP					
2	N^1-Oxide					
31	8-Hydroxy					

* Numbering and abbreviations as in Table I.

0.36	*0.33*	*0.30*	*0.27*	*0.24*	*0.21*	*0.18*	*0.15*
0.438							
0.262	0.400						
0.200	0.340						
0.219	0.352						
0.161	0.291						
0.066	0.199						
−0.043	0.086	0.202					
−0.056	0.075	0.200	0.338				
−0.189	−0.082	0.036	0.161				
−0.167	−0.060	0.075	0.202				
−0.286	−0.167	−0.039	0.131				
−0.286	−0.184	−0.031	0.148				
−0.156	−0.035	0.069	0.194				
−0.309	−0.172	−0.023	0.140				
−0.374	−0.258	−0.125	0.039	0.196			
−0.441	−0.301	−0.172	−0.004	0.163			
−0.462	−0.344	−0.207	−0.023	0.122			
	−0.374	−0.245	−0.073	0.082	0.232		
	−0.441	−0.309	−0.135	0.022	0.176		
	−0.452	−0.324	−0.172	−0.019	0.126		
	−0.533	−0.421	−0.258	−0.101	0.059		
		−0.441	−0.301	−0.156	−0.008	0.137	
	−0.546	−0.441	−0.279	−0.125	0.007		
			−0.411	−0.245	−0.091	0.069	0.225
			−0.452	−0.294	−0.161	0.004	0.176
			−0.520	−0.357	−0.213	−0.060	0.109
				−0.431	−0.324	−0.172	0.008
				−0.441	−0.316	−0.161	0.004
				−0.462	−0.332	−0.178	0.007
				−0.587	−0.452	−0.286	−0.109
				−0.568	−0.402	−0.245	−0.080
				−0.561	−0.401	−0.258	−0.091
				−0.757	−0.612	−0.430	−0.252
				−0.845	−0.688	−0.556	−0.392

TABLE IV

REGRESSION ANALYSIS OF THE RELATIONSHIP BETWEEN THE VOLUME FRACTION OF METHANOL, φ_M, AND LOG k': LOG k' = LOG $k_w - S\varphi_M$

The values in parentheses are defined in Table II.

*No.**	Substituent*	Log k_w	$-S$	r
8	2-*n*-Hexyl	2.95 (0.05)	4.49 (0.11)	0.999
24	8-PCTP	2.76 (0.04)	4.59 (0.09)	0.999
19	6-Benzyloxy	2.62 (0.04)	4.21 (0.08)	0.999
37	Dibutyryl	2.25 (0.05)	4.04 (0.10)	0.999
9	2-Phenyl	2.22 (0.05)	4.32 (0.10)	0.999
7	2-*n*-Butyl	2.18 (0.07)	4.25 (0.16)	0.998
32	2'-DNP	2.14 (0.05)	3.96 (0.10)	0.999
12	2-Thiopropyl	2.14 (0.04)	4.10 (0.10)	0.999
40	6-(S_p)-DMA-S	1.93 (0.03)	4.17 (0.07)	0.999
6	2-*n*-Propyl	1.72 (0.06)	4.05 (0.15)	0.997
39	6-(R_p)-DMA-S	1.62 (0.04)	3.89 (0.08)	0.999
20	6-Thiomethyl	1.56 (0.04)	3.76 (0.10)	0.998
17	6-DMA	1.54 (0.06)	3.83 (0.12)	0.998
5	2-Ethyl	1.52 (0.04)	4.37 (0.11)	0.999
11	2-Thiomethyl	1.43 (0.04)	4.10 (0.11)	0.999
21	Monobutyryl	1.42 (0.06)	3.73 (0.10)	0.998
23	8-Bromo	1.41 (0.06)	4.55 (0.17)	0.997
25	8-Thioethyl	1.41 (0.05)	4.76 (0.12)	0.999
36	(S_p)-cAMPS	1.35 (0.05)	5.02 (0.17)	0.998
16	6-MA	1.34 (0.09)	4.52 (0.26)	0.993
26	8-OH-i-P	1.33 (0.08)	4.79 (0.24)	0.995
38	1,N^6-Etheno	1.31 (0.08)	4.96 (0.25)	0.997
27	8-Methoxy	1.31 (0.02)	5.13 (0.08)	0.999
10	2-Chloro	1.29 (0.04)	4.06 (0.10)	0.999
35	(R_p)-cAMPS	1.27 (0.02)	5.22 (0.08)	0.999
18	6-Methoxy	1.20 (0.02)	3.76 (0.05)	0.999
15	6-Chloro	1.16 (0.02)	3.73 (0.06)	0.999
29	8-MA	1.15 (0.02)	4.87 (0.06)	0.999
1	cAMP	1.10 (0.04)	5.01 (0.13)	0.998
42	(S_p)-cGMPS	1.02 (0.01)	5.29 (0.06)	0.999
13	cPuMP	1.01 (0.01)	4.83 (0.05)	0.999
33	3'NH-cAMP	1.00 (0.04)	4.74 (0.13)	0.998
34	5'NH-cAMP	0.94 (0.02)	5.18 (0.11)	0.999
4	2-Methyl	0.88 (0.02)	5.18 (0.13)	0.999
22	8-Thio	0.77 (0.04)	5.20 (0.20)	0.997
28	8-Amino	0.74 (0.04)	4.97 (0.14)	0.998
30	8-DMA	0.73 (0.06)	4.90 (0.17)	0.994
14	cIMP	0.73 (0.01)	5.40 (0.08)	0.999
41	cGMP	0.68 (0.01)	5.18 (0.10)	0.999
3	N^1-Methoxy	0.68 (0.02)	5.33 (0.18)	0.998
2	N^1-Oxide	0.59 (0.01)	5.66 (0.10)	0.999
31	8-Hydroxy	0.35 (0.01)	4.97 (0.09)	0.999

* Numbering and abbreviations as in Table I.

where k' is the capacity factor of solutes j and i which differ by a substituent. When transformed to log k_w, eqn. 7 becomes

$$\tau_w = \log k_{w(j)} - \log k_{w(i)} \tag{8}$$

As log k_w and τ_w are directly related to the Gibbs free energy change attributed to the retention process[27,28], these retention parameters are equivalent, respectively, to log P and π of the standard Hansch approach[4,5]. cAMP was taken as the reference solute for all cyclic nucleotides, except for the C^6-substituted derivatives, which were related to the retention behaviour of purine 3',5'-monophosphate (cPuMP).

For C^2-substituted cAMP analogues that all carry hydrophobic substituents, including a congeneric series of alkylated derivatives (Table I), we observe a good correlation between τ_w and π, as is shown in Fig. 3A and described by eqn. 9:

$$\tau_w = 0.745\ (0.074)\ \pi - 0.366\ (0.076) \quad (9)$$
$$n = 9;\ r = 0.9671;\ F = 101;\ \text{S.D.} = 0.068$$

Similar mutual relations between τ_w and π have been described for simple model solutes[12]. For, *e.g.*, substituted benzene derivatives, however, a slope of the regression curve near unity and an intercept value close to zero have been observed[8,10–12], in-

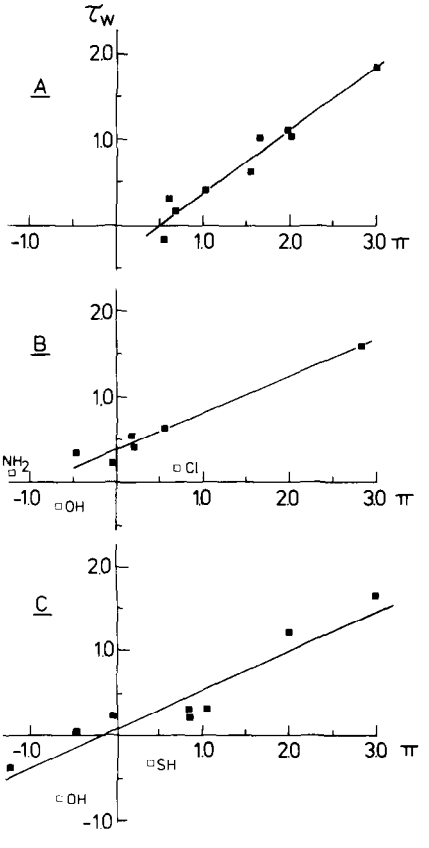

Fig. 3. Relationship between the group contribution constant to retention, τ_w, and the Hansch π constant[5]. (A) C^2-substituted nucleotides; (B) C^6-substituted nucleotides; (C) C^8-substituted nucleotides. Outliers are denoted by open squares and their substituents. The solid lines represent the regression lines according to eqns. 9–11.

dicating an almost identical behaviour of the solutes in both a liquid–liquid distribution and an RPLC system. This is obviously not the case for the C^2-substituted cAMP analogues. Owing to the low slope and the negative intercept value (eqn. 9), addition to the purine ring of a hydrophobic substituent, classified according to the Hansch π scale, enhances retention in our RPLC system to a much lesser extent than is predicted by the π value and may even result in reduced retention (see, *e.g.*, 2-CH_3-cAMP in Table IV).

The group of C^6-substituted analogues also includes strong electron-donating and electron-withdrawing substituents. When relating τ_w to π values of these compounds (Fig. 3B), a considerable scatter of the data points is observed. In seeking a linear relationship between the two parameters, three out of nine compounds have to be qualified as outliers to yield eqn. 10:

$$\tau_w = 0.424\ (0.051)\ \pi + 0.368\ (0.095) \qquad (10)$$

$$n = 6;\ r = 0.9723;\ F = 69;\ \text{S.D.} = 0.055$$

This manipulation may be justified by the following arguments. For cAMP (6-NH_2) and cIMP (6-OH), it is known that the additional electrons donated to the purine ring are effectively delocalized, resulting in different tautomeric forms of cAMP and cIMP in solution[29], which, by an increase in the (hydrophobic) surface area, enhance rather than reduce the overall hydrophobicity of the purine base. On the other hand, the apparent greater polarity of 6-Cl-cPuMP compared with its π value is explained by the large increase in the dipole moment of the base on Cl substitution (unpublished results), thus favouring solvation in the mobile phase.

Fig. 3C shows the relationship between τ_w and π for the C^8-substituted analogues. Again, two compounds had to be identified as outliers and hence had to be excluded from the regression analysis presented in eqn. 11:

$$\tau_w = 0.466\ (0.066)\ \pi + 0.108\ (0.186) \qquad (11)$$

$$n = 8;\ r = 0.9441;\ F = 49;\ \text{S.D.} = 0.117$$

Both outliers, 8-OH-cAMP and 8-SH-cAMP, are characterized by a high dipole moment and an electron distribution at C^8 and the neighbouring atoms, which favour at least partial ionization at neutral pH (unpublished results), thereby explaining the observed greater polarity of these compounds than is predicted by eqn. 11.

The relationships embodied in eqns. 9–11 and illustrated in Fig. 3 clearly substantiate the statement made in the Introduction that the π approach is not readily applicable to the description of the retention of complex structures where strong perturbing effects are exerted by substituents on the electrons of the heterocyclic ring. Although there is a reasonable mutual correlation between τ_w and π for most compounds, the magnitude of the contribution of substituents to retention is not *per se* predictable by considering their π values. We have therefore analysed whether a more general "bulk" parameter describing the geometric properties of a solute might be better able to represent the retention behaviour of cyclic nucleotides.

From the different parameters available, we have selected the molar refractivity (MR), which is directly related to the molar volume of a substituent[30]. MR is poorly correlated with π and values of MR for all substituents are readily available[5], making

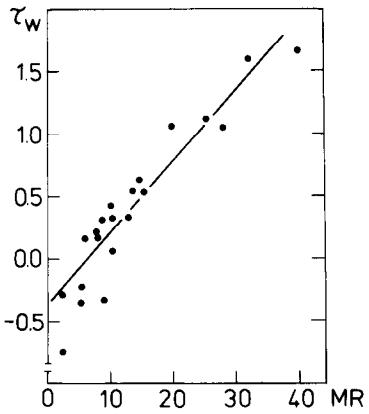

Fig. 4. Relationship between the group contribution constant to retention, τ_w, and the molar refractivity, MR, of substituents. Values of MR were taken from the compilation of Hansch and Leo[5]. The solid line represents the regression line according to eqn. 12.

possibly undue assumptions unnecessary. Fig. 4 illustrates the correlation between τ_w and MR, which is described by eqn. 12.

$$\tau_w = 0.062\ (0.005)\ \mathrm{MR} - 0.405\ (0.049) \qquad (12)$$
$$n = 24;\ r = 0.9383;\ F = 157;\ \mathrm{S.D.} = 0.013$$

Considering the crude nature of MR in describing "bulk" properties[30], the mutual agreement is remarkable, with only 12% of the variance in τ_w being unaccounted for by the model. Additionally, there is no need to treat each substituent position separately to yield a significant correlation.

The fact that the volume of a substituent is indeed an important determinant for retention illustrates the fundamental correspondence between retention in RPLC and liquid–liquid distribution in so far as both phenomena are governed by the solvophobic effect[27] and hence depend on the size of the cavity needed to incorporate a solute molecule into an aqueous phase. The inability of the Hansch π constant to describe adequately the retention of complex molecules is related to an inherent limitation of its use to such solute groups from which π values were derived. As the experimental determination of the distribution coefficients of charged cyclic nucleotides also suffers from great difficulties and uncertainties[6,8], we conclude that RPLC retention parameters may be well suited to describe quantitatively the hydrophobic nature of solutes, which is otherwise inaccessible to conventional techniques. Rather than attempt to seek correlations between log k_w and log P, it should be possible to use log k_w directly as a descriptor of the hydrophobicity of a solute.

The use of Table IV as a data source for studies on QSAR of cyclic nucleotides may be illustrated by the following example. The two diastereoisomers R_p-cAMPS and S_p-cAMPS (Fig. 1) act as antagonist and agonist of cAMP, respectively, in cAMP-dependent processes[31,32]. From the log k_w values in Table IV, it is apparent that the thioate analogues of cAMP possess a higher hydrophobicity than cAMP and should therefore be better able to pass cell membranes according to the τ_w values

for cAMP (0), R_p-cAMPS (+0.17) and S_p-cAMPS (+0.25). During our studies on the uptake of cAMP derivatives by a rat pheochromocytoma cell line, we found that the sequence with respect to the permeability of the membrane for these compounds was exactly as predicted by their log k_w values[33].

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