Biochimica et Biophysica Acta, 1061 (1991) 95–105
 0005-2736/91/$03.50
ADONIS 0005273691000511U

BBAMEM 75088

Nucleoside and nucleotide transport through a model liquid membrane. Periodic-catastrophic transport of a novel amantadine phosphoramidate conjugate of 5′-AMP

Donald E. Bergstrom [1], Julie K. Abrahamson [2] and Michael Y.-M. Chan [2]

[1] *Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, IN and* [2] *Department of Chemistry, University of North Dakota, Grand Forks, ND (U.S.A.)*

(Received 25 April 1990)
(Revised manuscript received 11 September 1990)

Key words: Nucleoside transport; Nucleotide transport; Periodic-catastrophic transport; Catastrophic transport; AMP; Amantadine; Phosphoramidate

Adenosine 5′-phosphor(adamantyl)amidate (5), an analog derived by linking the antiviral drug amantadine to 5′-AMP is transported through a model membrane system in a discontinuous periodic-catastrophic fashion. The system was composed of a glass cell containing two aqueous buffer phases separated by a chloroform layer. A more lipophilic, but structurally related derivative, adenosine 5′-phosphor(*n*-decyl)amidate (3) showed linear transport in the same system. Less lipophilic substances, including 5′-AMP and adenosine 5′-phosphor(morpholidyl)amidate (2), did not show transport. It is hypothesized that the periodic-catastrophic transport is a result of the collective activity of amidate 5 at the interface between the first aqueous interface and the chloroform layer. The time between catastrophic events is thought to be a reflection of the time necessary for molecular organization at the interface. The phenomenon is a new example of molecular organization in a system far from equilibrium leading to a repetitive dynamic process.

Introduction

The dynamics of molecular transport across cell-membrane lipid bilayers is an important process that has been studied in detail for small ions and some biologically active molecules [1]. Transport of electrically neutral small molecules across lipid bilayers is generally found to occur by a kinetically first-order process driven by the presence of a concentration gradient between the inner and outer aqueous phases. On the other hand, inorganic cation transport can occur by kinetically more complex periodic processes. Voltage oscillations are observed between model membrane separated aqueous phases, containing either different inorganic cations or a salt which is at greatly different concentrations in the two separated aqueous phases [2–4]. Because of experimental complexity, most studies have been done with model-membrane systems rather than with intact cells. These may be constructed from cell membrane material or be constituted from positively charged amphiphilic substances capable of forming liposomes, micelles, reverse micelles, or even simply a water-immiscible organic phase, such as chloroform.

Effective passive transport of small molecules across lipid bilayers requires that the molecules have an optimal balance between lipophilicity and hydrophilicity to maximize transport rate [5]. Stemming from our interest in designing transportable bioactive nucleotide analogs, we initiated a study to determine what type of lipophilic group could be attached at the 5′-phosphate to maximize transport rate, while maintaining water solubility. Our investigation eventually encompassed neutral C-5 substituted pyrimidine nucleosides structurally related to antiherpes agents [6–8], and phosphate modified adneosine structurally related to lipophilic nucleotide analogs of interest as antitumor agents [9–14]. Compounds from the latter group have been synthesized specifically to address the drug delivery and transportability problem inherent in underivatized nucleotides [15–17].

Abbreviations: BrU, 5-bromouridine; AU, absorbance units; UV, ultraviolet; THF, tetrahydrofuran; DMF, dimethylformamide; DMSO, dimethylsulfoxide.

Correspondence: D. Bergstrom, Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayett, IN 47907, U.S.A.

Materials and Methods

Analytical methods

Melting points were determined on a Büchi 510 apparatus. Infrared (IR) spectra were recorded in KBr pellets on a Nicolet MX-S spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Varian EM-390 spectrometer and are reported (d) downfield from either tetramethylsilane or sodium 3-(trimethylsilyl)-1-propane sulfonate as internal standards. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-260 spectrophotometer. Elemental analyses and mass spectral analyses were performed by Galbraith Laboratories, Knoxville, TN and Midwest Center for Mass Spectrometry at the University of Nebraska, Lincoln, respectively.

Column chromatography was carried out with the following packings: alumina 90 activated basic, 70–230 mesh; silica gel 60, 70–230 mesh (EM Reagents); DEAE Sephadex A-25 (Sigma); Bio-Gel P-2, 200–400 mesh; AG 50W-2S, 100–200 mesh, hydrogen form; Bio-Rex 70, 50–100 mesh, sodium form (Bio-Rad Laboratories). Analytical thin-layer chromatography (TLC) was done on the following plates: silica gel 60 F_{254}, 0.2 mm thick; alumina neutral (Type E) 60 F_{254}, 0.2 mm thick (EM Science); Whatman Cel 300 DEAE, 0.1 mm thick, Eastman Chromagram cellulose plate. Two solvent systems were used for thin-layer chromatography on silica gel: Solvent system A, 2-propanol/ammonia/water (7:1:2); Solvent system B, 1-butanol/acetonitrile/0.1 M ammonium hydroxide/ammonia (6:1:2:1). The thin layer plates were visualized under ultraviolet light.

Reagents and solvents

All chemicals used in the present investigation were reagent grade. Pyridine was stored over sodium in a constant reflux apparatus and distilled as needed. Tetrahydrofuran (THF) was dried by passing through an anhydrous alumina column, and then distilled from sodium/benzophenone before use. *N,N*-Dimethylformamide (DMF) was distilled from calcium hydride onto 4A molecular sieves. Dimethylsulfoxide (DMSO) was purified by vacuum distillation. Morpholine was distilled over sodium and stored in a bottle containing freshly-pressed sodium. Monoaza-18-*crown*-6(*crown* ether amine) was prepared as described in the literature procedure [18]. 1-Adamantanamine, *n*-decylamine and dicyclohexylcarbodiimide were purchased from Aldrich. Stearylamine and adenosine 5′-monophosphate were obtained from Sigma. *t*-Butyl alcohol was purified by distillation over calcium oxide and only the center portion was collected. Other commercially unavailable compounds were prepared in this laboratory, fractionally distilled or subjected to column chromatography until they were pure. The syntheses of the C-5 alkyl deoxyuridines have been previously reported [19]. 5-Bromouridine was prepared by direct bromination of uridine in DMF following the procedure of Duval and Ebel [20].

Synthesis of adenosine 5′-phosphoramidates

The nucleoside 5′-phosphoramidates were synthesized by the procedure of Moffat and Khorana [21]. A detailed procedure is illustrated for adenosine 5′-phosphor(adamantyl)amidate (**5**). Products were purified by anion-exchange chromatography on DEAE Sephadex A-25. Two solvents systems were used for gradient elutions: (i) aqueous ammonium bicarbonate varying in concentration from 0.005 M to 0.1 M; (ii) 0.005 M ammonium bicarbonate/ethanol (3:7) to 1.0 M ammonium bicarbonate/ethanol (4:6). Solvent (i) and (ii) were used to separate water-soluble and water-insoluble phosphoramidates from 5′-AMP, correspondingly. A cation exchanger was used to convert the desired phosphoramidate to a sodium salt form. Dowex 50W-8X in H^+ form was changed to sodium form by washing the Dowex resins sequentially with deionized water, 1.0 M sodium hydroxide and deionized water, until the aqueous eluate showed a pH 6.0–8.0. The phosphoramidates were converted to the sodium salt on Dowex 50W-8X (Na^+) by loading them onto the resin and eluting with deionized water until the phosphoramidate was completely eluted. Finally, the phosphoramidates were passed through a Bio-Gel P-2 column eluting with deionized-distilled water to remove inorganic salts. Identity and purity were established by ^{1}H, FAB mass spectra, UV, TLC and elemental analysis.

Adenosine 5′-phosphor(morpholidyl)amidate (2)

The procedure reported by Moffatt and Khorana was followed. $R_F = 0.56$ (solvent A); $R_F = 0.276$ (solvent B); ^{1}H-NMR (D_2O) δ 8.30 (1H, s), 8.00 (1H, s), 5.98 (1H, d, $J = 5$ Hz), 4.46 (1H, t, $J = 5$ Hz), 4.30 (m), 4.02 (2H, m), 3.50 (4H, t, $J = 4$ Hz), 2.90 (4H, t, $J = 4$ Hz); MS: *m/e* 437 M^+, 415 (anion); UV(H_2O), λ_{max} 259.6 nm (ϵ 14420). Elemental analysis calculated for $C_{12}H_{20}N_6O_7PNa \cdot 2.5H_2O$:
Calcd.: C, 34.78; H, 5.17; N, 17.39.
Found: C, 34.70; H, 4.85; N, 17.20.

Adenosine 5′-phosphor(n-decyl)amidate (3)

The procedure was similar to the one for the preparation of adenosine 5′-phosphor(adamantyl)amidate. The adenosine 5′-phosphor(*n*-decyl)amidate was isolated as its ammonium salt, (345 mg; 34.3% yield). $R_F = 0.359$ (B); $R_F = 0.674$ (A); ^{1}H-NMR ($D_2O/CD_3OD = 1:3$) δ 8.60 (1H, s), 8.23 (1H, s), 8.19 (1H, s), 6.09 (1H, d, $J = 5$ Hz), 4.44 (1H, m), 4.28 (2H, m), 4.01 (1H, m), 2.94 (impurities), 1.125–1.37 (18H, m) 0.83 (3H, m); UV (95% EtOH), λ_{max} 260 nm (ϵ 7460); MS, 485 $M - (C_{20}H_{34}N_6O_6P$ anion).

Adenosine 5'-phosphor(1,4,7,10,13-pentaoxo-16-azacyclo-octadecyl)amidate (4)

A procedure similar to that described below for compound **5** was followed. Following anion-exchange chromatography, one single spot on a silica gel plate by TLC analysis confirmed the purity of the desired product. $R_F = 0.29$ (B); $R_F = 0.54$ (A); ^{1}H-NMR (D_2O) δ 8.44 (1H, s), 8.18 (1H, s), 6.05 (1H, d, $J = 5$ Hz), 3.00–3.75 (48H, m); 1.0–2.0 (16H, m); UV(H_2O), λ_{max} 259.8 nm (ϵ 13500).

Adenosine 5'-phosphor(ad..mantyl)amidate (5)

1-Adamantanamine (0.605 g, 4 mmol) was added to a solution of 50% aqueous *t*-butyl alcohol (20 ml) containing adenosine 5'-phosphate (0.365 g, 1.0 mmol). The suspension changed to a clear solution as refluxing began. Dropwise addition of dicyclohexylcarbodiimide (0.825 g, 4.0 mmol) in *t*-butyl alcohol (10 ml) to the refluxing solution was completed in 2 h. After refluxing for 22 h, the reaction mixture was cooled to room temperature. The white crystalline precipitate was separated by suction filtration and washed several-times with *t*-butyl alcohol. The residue was suspended in a mixture of ethanol/water (7 : 3) and filtered. The aqueous ethanol solution was reduced to a minimum volume which was then subjected to anion-exchange chromatography.

A 0.6 g sample of reaction mixture was applied onto a column (1.65 cm diameter × 30 cm height) of DEAE Sephadex A-25. Elution was begun, using a linear gradient of ammonium bicarbonate in ethanol. The mixing vessel initially contained 450 ml of ethanol/0.005 M ammonium bicarbonate (7 : 3) and the reservoir 450 ml of ethanol/1.0 M ammonium bicarbonate (6 : 4). The fractions collected were analysed by TLC. The fraction (112–144 ml) with an $R_F = 0.337$ (B) was concentrated and last traces of ammonium bicarbonate were removed by several further evaporations after addition of small amounts of water. Finally, 239 mg of the ammonium salt of **5** (48% yield) was obtained after lyophilization:

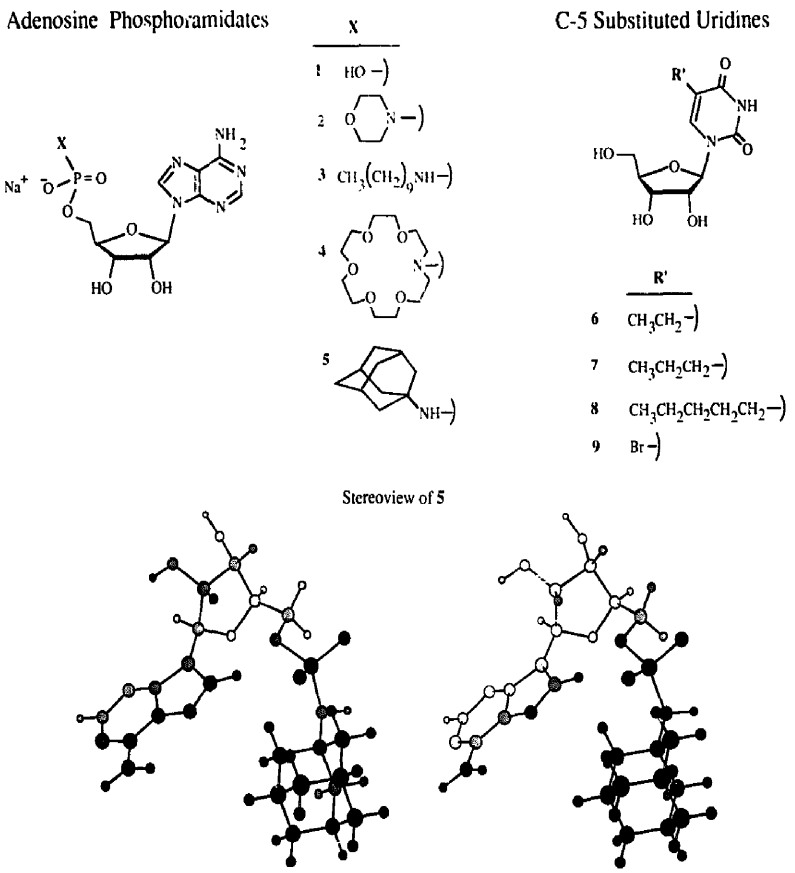

Fig. 1. Nucleosides and nucleotides.

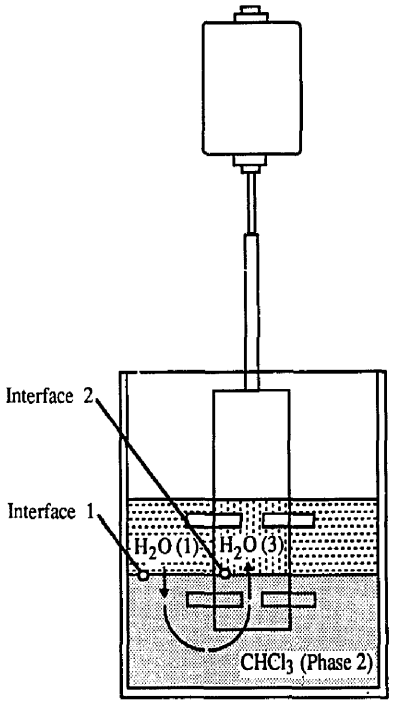

Fig. 2. Model transport apparatus. The area of interface 1 was 12.8 cm^2, and area interface 2 was 3.8 cm^2.

$R_F = 0.64$ (A); $R_F = 0.348$ (B); ^{1}H-NMR (D_2O) δ 8.50 (1H, s), 6.11 (1H, d, $J = 4.5$ Hz), 4.32 (1H, m), 3.98 (2H, m), 1.34–1.85 (15H, m); MS. 479 M^- ($C_{20}H_{28}N_6O_6P$ anion); UV (H_2O), λ_{max} 259.6 nm (ϵ 12900). Elemental analysis calculated for $C_{20}H_{32}N_7O_6P \cdot 5H_2O$:

Calcd.: C, 40.88; H, 7.15; N, 16.69.

Found: C, 40.44; H, 6.68; N, 16.76.

The three-dimensional representation of **5** (Fig. 1) was created in the software program Chem 3D (Cambridge Scientific) by incorporating the parameters found for adenosine in uridylyl 3′,5′-adenosine phosphate [21].

Transport apparatus

The apparatus (Fig. 2) (Den Norsk Glassblåsende Fabrikk) for measuring transport through a $CHCl_3$ liquid membrane was constructed using a 20 ml beaker in which was suspended a glass tube (22 mm i.d.) connected to a stirring motor (either a 3/8″ variable speed reversible drill or a 60 rpm constant speed motor) mounted on a ring stand. Three pairs of 2 mm glass rods (7–9 mm long) were fused perpendicularly to the tube: one pair at the bottom on the outside, a second pair about 5 mm above the first pair, and a third pair on the inside at the height of the upper outer rods. A hole was cut in the side of the central tube to permit access to the inner aqueous solution using pipets. The beaker was clamped in position to allow 2–3 mm between the bottoms of the tube and the beaker. The $CHCl_3$/aqueous-buffer interface fell between the pairs of outer rods on the tube as shown in the figure. When the drill was used, the rate of the rotation of the tube, and thus of the stirring in the system, was controlled using a variable transformer with the drill locked in high speed. Measured stirring rates with the drill were not constant, but were usually 40–55 rpm, and ranged from 30 to 90 rpm. In this apparatus, the $CHCl_3$ layer was added first using a 20 ml glass syringe, followed by 1000 μl of the inner aqueous-buffer solution (using an adjustable pipet), then the entire outer solution (using a 10 ml glass syringe), and lastly the remainder of the inner aqeous solution. The aqueous-buffer solution used in all experiments was 10 mM potassium phosphate (pH 7.3), 0.16 M NaCl. The volumes of the three phases were: aqueous phase 1, 7.0 ml; aqueous phase 2, 3.0 ml; and chloroform (phase 2), 12 ml. Transport was measured by monitoring the absorbance of aliquots from the inner aqueous layer after various stirring intervals. The aliquots were returned to the system following the measurement. Absorbance measurements were made on a Shimadzu UV-260 UV-Vis spectrometer in semimicro (4 mm wide × 10 mm pathlength) quartz cuvettes using the same aqueous buffer as a reference. Stirring was halted during the times the absorbance was measured.

Extraction coefficients

Extraction constants between aqueous buffer and chloroform were determined. Nucleotides were prepared in a solution of 10 mM potassium phosphate (pH 7.3), 0.16 M NaCl. The absorbance of each solution was scanned to determine the wavelength and intensity of the maximum. Molar extinction coefficients (ϵ) were calculated for each compound. 2.0 ml of the nucleotide solution was mixed with an equal volume of $CHCl_3$ in a large test-tube. The mixtures were extracted five-times for 1 min using a vortex mixer with 1 min pauses between extractions. The phases were allowed more than 10 min to equilibrate, and the absorbance of the aqueous phase was measured. The extraction coefficient, K, which is defined as $K = C_o/C_w$, where C_o is the concentration of the substance in chloroform at equilibrium and C_w is the concentration in the aqueous phase at equilibrium was calculated as follows:

$$K = \frac{[A(\text{before}) - A(\text{after})]}{[A(\text{after})]}$$

where A is the absorbance measured at the maximum before and after $CHCl_3$ extraction. Each value represents the average of at least one set of duplicate extractions.

Results

The analogs chosen for our study included four phosphoramidates derived from AMP, three C-5 alkyluridines (Fig. 1), and 5-bromouridine. The simplest system for measuring transport rates, and yet which can be extrapolated to more complex lipid bilayers, is a model membrane constructed of aqueous phases separated by an immiscible organic layer such as chloroform. The system (Fig. 2) was designed so that all three layers were stirred. Stirring was setup to minimize disruption of the total system, but yet keep the bulk phases homogeneous. One could observe shear near the interface, implying that there is a relatively undisrupted quiescent layer where the process of molecular movement is probably diffusion controlled. The question of artifacts introduced by stirring can not be unequivocally answered. Neither stirring nor sampling appeared to be directly related to the transport phenomena. The rate of transport was determined spectrophotometrically, as described in Materials and Methods, by measuring the appearance of the nucleoside in aqueous phase 3. The stirring was discontinued during the period of time that it took to withdraw a sample for spectrophotometric determination. Following the absorbance measurement, the sample was returned to the cell and stirring recontinued. Experiments with the alkyl uridines in which the number of assays per unit time varied considerably (four versus sixteen data points over a period of 80 min; Fig. 3) showed that sample withdrawal did not introduce an observable error. The transport studies were carried out for a maximum of 400 min. The rate of solvent evaporation was relatively slow, but noticeable over time periods longer than 400 min, thereby precluding us from continuing experiments for longer periods of time.

When the three C-5 substituted nucleosides **6**–**8** were allowed to migrate from aqueous phase 1 to aqueous phase 3, the increase in absorbances for each compound was linear over the short time span of the experiment (Fig. 3). Reproducibility between experiments was generally good, as illustrated in the figure. The respective relative rates of transport for **6**, **7**, and **8** were 1 : 7 : 39. A detailed series of experiments with another C-5 substituted nucleoside, 5-bromouridine (**9**) established that transport followed pseudo-first-order kinetics on application of a steady-state transport model. Data for 5-bromouridine (BrU) transport is shown in Table I. The rate of transport of BrU across a phase boundary should be dependent on the surface area of the interfaces, as well as on the concentration of BrU; hence, these two parameters were varied in rate studies. The concentrations of BrU in the three phases are indicated, respectively, by $[BrU_1]$, $[BrU_2]$, $[BrU_3]$. Relatively consistent values for k_1 could be obtained by using a steady state approximation, wherein $d[BrU_2]/dt$ was assumed to be very small and, hence, could be approximated as zero. Over the course of the experiment, $[BrU_1] \gg [BrU_3]$. Therefore, terms containing $[BrU_3]$ are neglected.

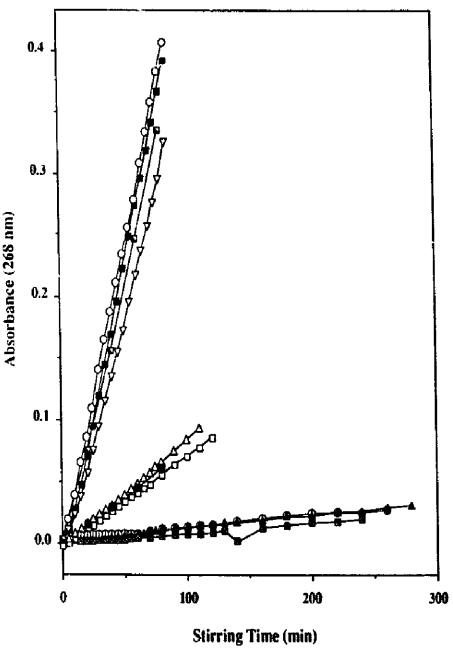

Fig. 3. Rate of appearance of the C-5 substituted uridine derivatives 5-ethyluridine (**6**), 5-propyluridine (**7**), and 5-pentyluridine (**8**) in the inner aqueous phase (cf. Fig. 2) as measured by the increase in absorbance at 268 nm. The absorbance in aqueous phase 2 is plotted versus the stirring time of the system. Three separate experiments were carried out with each of the nucleosides 5-ethyluridine (⊠, ⊕, and ▲) and 5-propyluridine (□, ●, △). Four separate experiments are shown for 5-pentyluridine (▣, ○, ■, ▽).

TABLE I

Dependence of 5-bromouridine transport on concentration and interface area

$[BrU_1]$ = Concentration of 5-bromouridine in aqueous phase 1; A_1, the area of the interface between aqueous phase 1 and chloroform (phase 2); A_2, the area of the interface between aqueous phase 3 and chloroform (phase 2).

$[BrU_1]$ ($M \times 10^4$)	A_1 (cm^2)	A_2 (cm^2)	$d[BrU_3]/dt$ (M/min)	k_1 ($min^{-1} \cdot cm^{-2}$)
5.0	12.8	3.8	$2.88 \cdot 10^{-6}$	$4.5 \cdot 10^{-4}$
5.0	12.8	3.8	$3.04 \cdot 10^{-6}$	$4.8 \cdot 10^{-4}$
1.0	12.8	3.8	$4.86 \cdot 10^{-7}$	$3.8 \cdot 10^{-4}$
1.0	3.8	12.8	$1.62 \cdot 10^{-7}$	$4.3 \cdot 10^{-4}$
3.3	3.8	12.8	$5.36 \cdot 10^{-7}$	$4.3 \cdot 10^{-4}$

According to the steady state approximation,

$$k_1[BrU_1][A_1] - k_{-1}[BrU_2][A_1] - k_2[BrU_2][A_2] = 0$$

where k_1 is the rate constant for the transport of BrU from aqueous phase 1 to chloroform (phase 2) across interface 1 of area A_1. k_{-1} is the rate constant for the transport in the opposite direction across the same interface, and k_2 is the rate constant for the transport of BrU from chloroform (phase 2) to aqueous phase 3, across the second interface with area A_2.

Then

$$[BrU_2] = \frac{k_1[BrU_1][A_1]}{k_{-1}[A_1] + k_2[A_2]}$$

while the

$$\text{rate} = k_2[BrU_2][A_2]$$

Substituting for $[BrU_2]$ in the rate expression yields:

$$\text{rate} = \frac{k_1 k_2[A_1][A_2][BrU_1]}{k_{-1}[A_1] + k_2[A_2]}$$

If the transport across the first interface is rate determining, then

$$k_2[A_2] \gg k_{-1}[A_1]$$

hence

$$k_{-1}[A_1] + k_2[A_2] \approx k_2[A_2]$$

or

$$\text{rate} = \frac{k_2 k_2[A_1][A_2][BrU_1]}{k_2[A_2]} = k_1[A_1][BrU_1]$$

Using this approximation, the value of k_1 was found to yield fairly closely matching values in two sets of experiments ($(4.4 \pm 0.6) \cdot 10^{-4}$ $min^{-1} \cdot cm^{-2}$) and ($(4.3 \pm 0.1) \cdot 10^{-4}$ $(min^{-1} \cdot cm^{-2})$) (see Table I). k_1 was determined to be independent of concentration, since transport at three different concentrations (1.0, 3.3 and $5.0 \cdot 10^{-4}$ M) gave similar calculated values when the steady state approximation was used.

The area of the interfaces between the phases was varied simply by reversing the aqueous phase in which the sample was introduced. In the experiments in which A_1 was 12.8 cm^2 and $[BrU_1]$ was 10^{-4} M, the rate of transport was 3 times greater than the experiment in which A was 3.8 cm^2 and $[BrU_1]$ 10^{-4} M. Since the ratio of areas is $12.8/3.8 = 3.4$, agreement is fairly good. The other experiments listed in Table I are also in agreement. Another set of experiments was attempted using the same inner apparatus, but a different outer vessel so that A_1 was 24.7 cm^2, and A_2 remained at 3.8 cm^2. Although one experiment was in very close agreement ($k_1 = 4.9 \cdot 10^{-4}$ $(min^{-1} \cdot cm^{-2})$), the system was generally less reproducible.

TABLE II

	Compound	Extraction coefficient K	Relative transport rate
1	AMP	0.012	0
2	Adenosine 5′-phosphor-(morpholidyl)amidate	0.0104	0
3	Adenosine 5′-phosphor-(*n*-decyl)amidate	0.144	9
4	Adenosine 5′-phosphor-(1-aza-18-*crown*-6)amidate	0.021	0
5	Adenosine 5′-phosphor-(adamantyl)amidate	0.034	1–16 (variable)
6	5-Ethyluridine	0.0118	1
7	5-Propyluridine	0.0130	7
8	5-Pentyluridine	0.0319	39

Finally, one experiment using a different inner apparatus ($A_1 = 2.5$ cm^2, and $A_2 = 12.8$ cm^2) yields a value of $k_1 = 5.0 \cdot 10^{-4}$ $(min^{-1} \cdot cm^{-2})$, which again was within experimental error.

Overall, these experiments illustrate that transport follows pseudo-first-order kinetics, and that when transport occurs from aqueous phase 1 to aqueous phase 3, the area of the first interface is rate controlling. These results are consistent with the first order kinetic behavior which is predicted from Fick's laws of diffusion and mass transfer [23].

The relative rates of transport of **6**, **7**, and **8** corresponded to lipophilicity as measured by extraction coefficients (Table II). The most lipophilic derivative, 5-pentyluridine showed the highest rate of transport, while the least lipophilic, 5-ethyluridine clearly showed less rapid transport.

In contrast to the C-5 alkyluridines, the transport behavior of the phosporamidates of similar lipophilicity (as measured by extraction coefficients; cf. Table II) was far less consistent. In most experiments AMP (**1**), adenosine 5′-phosphor(morpholidyl)amidate (**2**) and adenosine 5′-phosphor(1-aza-18-*crown*-6)amidate (**4**) did not show transport. However, one experiment with **2** showed transport behavior similar to that described below for adenosine 5′-phosphor(adamantyl)amidate (**5**). In three experiments adneosine 5′-phosphor(*n*-decyl)amidate (**3**) gave fairly linear transport rates, one example of which is plotted in Fig. 4, along with the data for nucleotide **5**.

The transport system is quite sensitive to the presence of other substances. A series of experiments with ϵAMP diesters was discarded because difficult-to-remove impurities were apparently transported faster than the ϵ derivatives. Failure to carefully clean glassware (a final scrubbing with an anionic detergent and adequate

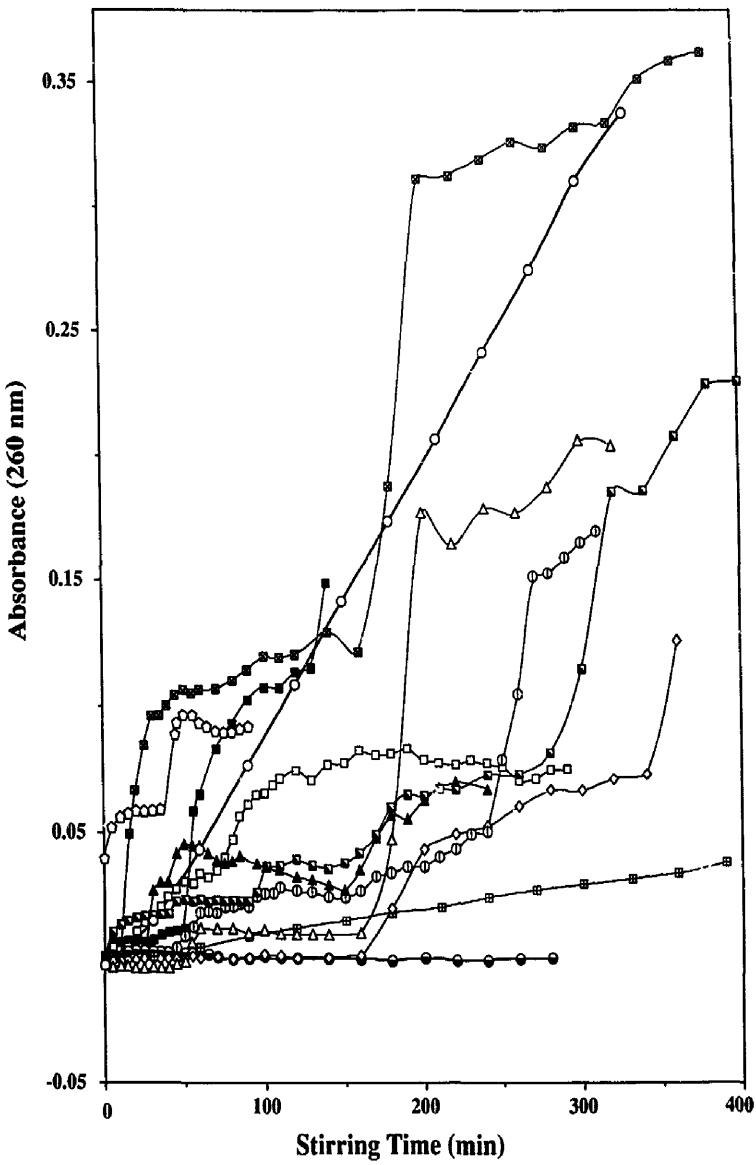

Fig. 4. The absorbance in aqueous phase 3 was plotted versus the stirring time of the system for adneosine 5′-phosphor(adamantyl)amidate initially placed in aqueous phase 1 at a concentration of $5 \cdot 10^{-4}$ M. Ten separate experiments are shown. For comparison, experiments with adenosine 5′-monophosphate (**1**), which gave virtually no transport (●), and with adenosine 5′-phosphor(*n*-decyl)amidate (**3**), which resulted in nearly linear transport (○), are shown. The curves connecting the points were generated by a Steinman interpolation routine.

rinsing with distilled water is necessary) gave apparent spurious results in other expriments.

Although nucleotides **1**, **2** and **4** all showed extraction coefficients similar in magnitude to that determined for 5-ethyluridine, they did not show transport. It is unlikely that the extraction coefficients determined for the highly hydrophilic amphiphilic compounds are true extraction coefficients because of absorption of the molecules at the water/$CHCl_3$ interface or on the glass surfaces. A simple calculation, assuming, (1) that each molecule occupies from 50 to 100 nm^2 of surface area, (2) that the surface area between the H_2O

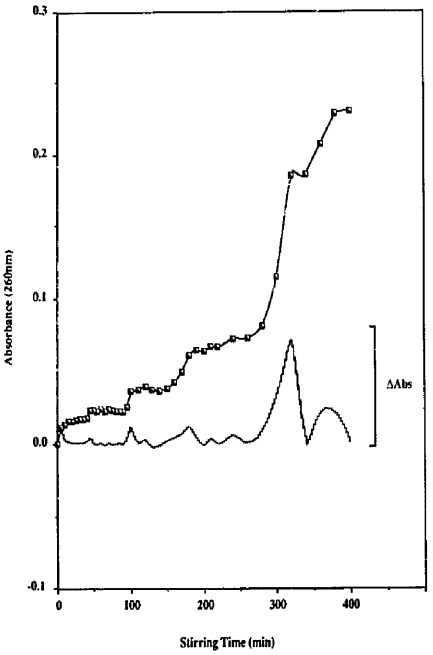

Fig. 5. The absorbance in aqueous phase 3 is plotted versus the stirring time of the system for one representative experiment with adenosine 5′-phosphor(adamantyl)amidate under the conditions specified in Figs. 2 and 4 (■). The curve connecting the points was generated by a Steinman interpolation routine. The lower curve shown in the figure is the first derivative of the upper curve and graphically represents the rate at which the phosphoramidate passes from aqueous phase 1 to aqueous phase 3.

and $CHCl_3$ layers in the extraction experiment is approx. 10 cm^2, and (3) that the compounds have extinction coefficients in the range $(1.2–1.7) \cdot 10^4$ M^{-1}, yields decreases in absorbance of 0.008 to 0.027 from 2 ml of aqueous solution. This is clearly within the range of the absorbance decreases that we measured in our extraction experiments.

Unlike the other derivatives, adenosine 5′-phosphor (adamantyl)amidate (**5**) showed periodic-catastrophic transport in most experiments (Fig. 4). We have deemed the observed transport 'catastrophic', because it shows behavior typical of systems far from equilibrium which reach bifurcation points that lead to sudden changes in the state of the system [23]. The transport is periodic with multiple catastrophic events typically occurring within the 300-min time span of most experiments.

The periodic aspect of the transport varied from experiment to experiment and can be best discerned by examining one experiment in detail. At least six catastrophic events in a very distinct pattern can be observed in one experiment (Fig. 5). After an initial rapid transition had occurred within 5 min (0.0116 AU) six additional transitions occurred at 45 (0.0048), 100 (0.0106), 170 (0.0268), 230 (0.0056), 300 (0.113) and 350 (0.042) min. The time interval between major transitions falls in the range 50 to 70 min with the exception of the early transitions (5 and 45 min). Close inspection of the graph (Fig. 5) indicates that each major transition is followed closely by a transition of much smaller magnitude. Following some transitions there are alternating decreases and increases in absorbance that resemble resonance ringing effects, as if the system goes too far in one direction and must rebound in the opposite direction. The phosphoramidate must be in the sodium salt form. A series of experiments with tetrabutylammonium cation added to aqueous phase 1 enhanced the rate of transport of phosphoramidate **5** and completely suppressed periodic-catastrophic transport. The transport appeared linear over time in these experiments.

Discussion

Three distinct types of transport phenomena were observed with three distinct types of molecules treated under the same conditions. AMP did not transport, neutral nucleoside analogs transported with pseudo-first-order kinetics, and phosporamidate **5** transported in periodic catastrophic bursts. This implies that the phenomenon is a molecular level phenomenon and not an apparatus contrived artifact. For compound **5**, two aspects of the transport, the magnitude of the individual transitions and the period between transitions, warrant discussion. For the ten experiments with **5** shown in Fig. 4, the magnitude of the transitions (absorbance change) was plotted versus occurrence (Fig. 6). There appear to be some transition ranges slightly more prevalent than others. The smallest transition distinguishable above background noise was about 0.002 absorbance units. Specifications for the ultraviolet spectrophotometer used in this study indicate photometric repeatability of ± 0.001 A in the range 0 to 0.5 A, hence, transitions involving changes in absorbance less than this could not be determined. At least five transitions occur in the region 0.0025 ± 0.0005 AU. Six transitions occur with a magnitude of 0.0050 ± 0.0005 AU. Other transition plateaus are apparent at 0.012 ± 0.001 AU (six transitions), 0.027 ± 0.03 AU (six transitions), and 0.043 $\pm$ 0.004 AU (five transitions).

We wondered whether these numbers were meaningful in terms of how molecules transport in a catastrophic manner. Without additional detailed knowledge, it is difficult to say how these molecules may be packaged into three-dimensional transportable arrays. However, we can determine the number of molecules transported per transition and speculate from there. From the molar absorptivity of **5** ($\epsilon = 12\,900$) and the volume of aqueous phase 3 (3.0 ml), the number of molecules per 0.0001 AU can be calculated to be $(0.003\ l) \times (1/12\,900$

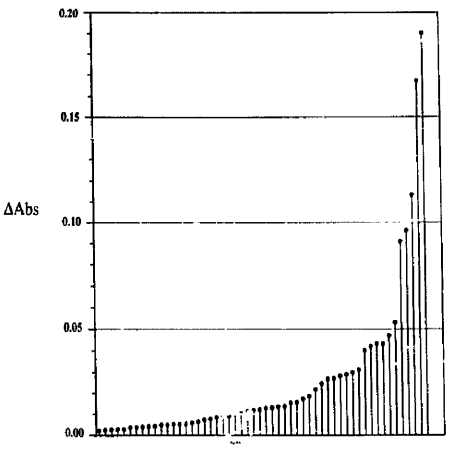

Fig. 6. Magnitudes of absorbance increases in aqueous phase 3 during transport of **5**. All transitions of magnitude greater than 0.0020 absorbance units (AU) from the ten adenosine 5′-phosphor(adamantyl)amidate experiments shown in Fig. 4 have been compiled and are depicted in order of increasing magnitude.

$M^{-1}) \times (0.001\ AU) \times (6.02 \cdot 10^{23}\ molecules \cdot mol^{-1}) = 1.4 \cdot 10^{13}$ molecules/0.0001 AU. From this data, we can determine how many molecules are transported in any given transition. For example, the transitions with $A = 0.012 \pm 0.001$ result from transport of $1.7 \cdot 10^{15}$ molecules. Since we believe that interface absorption may be a prerequisite to catastrophic transport, it is of interest to compare this number to the number of molecules potentially absorbable on the interface between aqueous phase 1 and the chloroform. Measurements of CPK models suggests a minimum surface area of about 70 $Å^2$/molecule (0.7 nm^2). The number of molecules capable of packing into a monolayer at interface 1 (12.8 cm^2) would theoretically be about $(12.8 \cdot 10^{14}\ nm^2)/0.7\ nm^2 = 1.8 \cdot 10^{15}$. This number is strikingly similar to that calculated above for the 0.012 AU transition.

Heretofore, there has been no indication that one could build into a single molecule the structural elements necessary for catastrophic transport to occur at an interface. All previous systems known to undergo oscillatory transport have involved inorganic ions in conjunction with interfaces. The model membranes may be cell-membrane material or be constituted from positively charged amphiphilic substances capable of forming liposomes, micelles or reverse micelles, or even simply a water immiscible organic phase such as chloroform. In these systems, the key role is not played by the inorganic ion which yields the oscillatory voltage response, but by the lipid bilayer that undergoes phase transitions [24], or by an organic amphiphile that undergoes the organization process necessary to entrap, transport, and release the ion. Our system also appears to be quite different from the chemically driven oscillatory system responsible for a Marangoni effect described by Duprerat and Nakache, since we are not generating different chemical species at the interface [25].

How does adneosine 5′-phosphor(adamantyl)amidate (**5**) organize in order to cross the interface? Studies on structural requirements for molecular association would suggest that **5** is not a good candidates for micelle, reverse micelle or liposome formation [26]. The lipophilic portion of the molecule can not obtain the critical length (cf. three dimensional representation of **5** in Fig. 1) necessary for packinging into any of these forms. Also osmotic coefficient data [27] on adenosine suggests that very little association between purine rings would occur in aqueous solution ($K = 4.5\ M^{-1}$) at the concentrations employed in our experiments. Association between molecules could be enhanced at an interface, since orientation of the molecules at the interface might place the adenine moieties in appropriate positions for base stacking to occur.

In order to determine whether the 5′-phosphor(adamantyl)amidate (**5**) was transporting in an associated structural form that included aqueous solution, we carried out two experiments in which pyrenetetrasulfonic acid, tetrasodium salt was added to aqueous phase 1 at a concentration of $1.24 \cdot 10^{-4}$ M. The first experiment included only buffer and the pyrenetetrasulfonate. After 2 h, the change in absorbance at 375 nm in phase 3 was 0.007 AU.

In the second experiment, $5 \cdot 10^{-4}$ M **5**, $1.24 \cdot 10^{-4}$ M pyrenetetrasulfonic acid, tetrasodium salt were combined in aqueous phase 1. In this case, the change in absorbance at 375 nm was 0.038 AU. Nucleotide **5** showed two catastrophic transitions, centered at 35 min and 65 min. The pyrenetetrasulfonate transport was nearly linear and did not parallel the catastrophic events observed for **5** in the same solution.

This experiment suggests that **5** may have slightly enhanced the rate of transport of pyrenetetrasulfonate, but that the effect is general rather than specific. That is, during the period of catastrophic transport structures that occlude aqueous solution are probably not formed.

We speculate that the following occurs: (1) In a slow step **5** is randomly absorbed at the interface between aqueous phase 1 and the chloroform with the adamantyl moiety projecting into the chloroform layer and the ionic phosphoramidate group projecting into the aqueous phase. This arguably may be the thermodynamically most stable location for these molecules. (2) At some point an interface packing limit is obtained and the systems becomes unstable. The shape of the molecules implies that packing interactions between them could lead to stress in the form of surface curvature. Eventually, crowding of additional molecules into the monolayer may lead to either collapse of the monolayer

discharging **5** into the chloroform phase in an unknown aggregated form or to higher dimensional structures (e.g., layers folding back on themselves) which remain at the interface. (3) Driven by the osmotic pressure difference between the aqueous phases. **5** (or some unknown aggregates of **5**) migrate through the chloroform layer to interface 2. (4) The molecules are absorbed at interface 2. (5) Molecules diffuse from the interface into aqueous phase 2. Transport across the second interface may or may not occur as aggregates. The low concentration of **5** in aqueous phase 3 and its high solubility in water may favor rapid diffusion into the aqueous phase 3. The effects of the unstirred boundaries at the interfaces [33] on our simplified model are unknown. Concentration gradients at these boundaries are possible and this could certainly play a role in the way in which molecules become organized at the interface. We have also neglected effects due to specific chloroform interactions with phosphoramidate **5**, a problem which should be addressed in future studies.

It is noteworthy that among the AMP phosphoramidates included in this study, the only molecule that routinely showed catastrophic transport was a conjugate of amantadine (1-adamantanamine), a drug which prevents influenza A2 and is known to enter cell membranes [29]. The influences of amantadine (induction of phase separation, transition temperature lowering and increased bilayer fluidity) and related derivatives on bilayer membranes have been extensively studied [30]. The absorption of amantadine at lipid bilayers has been measured and a surface density of one molecule per 300 $Å^2$ with $K = 1.3 \cdot 10^4\ M^{-1}$ determined [31].

With this information and our experimental result, we can speculate further about how and why these molecules achieve catastrophic transport. Neutral (uncharged molecules) with some lipophilicity can migrate into the chloroform layer unimpeded by a large energy barrier. Hence, one sees a continuous leakage of individual molecules across the interface into the second aqueous phase. Charged hydrophilic molecules (e.g., AMP) with little affinity for the organic layer do not readily bind at the interface. 5′-Phosphor(*n*-decyl) amidate (**3**) which showed apparent pseudo-first-order transport kinetics is significantly more soluble in chloroform than any of the other derivatives examined in this study (Table II), yet it migrated at a significantly lower rate than the other phosphoramidates between the two aqueous phases. This effect may reflect a rate controlling organization into aggregates (such as reverse micelles) in order to achieve transport. Adenosine 5′-phosphor(adamantyl)amidate (**5**), with its compact globular lipophilic attachment is unlikely to aggregate very well becauseof the small hydrocarbon surface area. In addition, because ionic molecules greatly favor the aqueous phase, the transport of individual molecules into the chloroform layer must have a high energy barrier. However, we speculate the certain types of amphiphilic molecules like **5**, can be absorbed at the interface, and when the interface surface is filled, they now act as a collective organization in which perturbations at one point on the surface are rapidly transmitted to distant points on the surface. As a collective whole, they can garner the energy necessary to traverse the barrier and transport into the chloroform layer. This effect requires a closed system with distinct boundaries in order to contain the molecules and allow assembly into a collective whole.

Given the complexity and specificity of surface interaction phenomena [32], the picture of transport that we have constructed at this point is conjectural. At this point no attempts to mathematically model the periodic-catastrophic transport have been attempted. Future studies to include measurements of interface adsorption isotherms, changes in electric potential and the influence of molecular structure on the phenomenon will be necessary in order to develop a clearer picture of what may actually be occurring at the molecular level.

Acknowledgements

The National Institutes of Health is gratefully acknowledged for support of this research through NIH Grant GM30805. Mass spectral determinations were performed by the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Instrumentation Facility (Grant No. CHE 8211164). We gratefully acknowledge NSF Instrument Grant CHE-8509872 for contributing to the purchase of the VXR 300 NMR spectrometer. The technical support of John Bergstrom and Pamela Sakkinen, who independently re-checked many of the transport experiments, and Dan Swartling of Den Norsk Glassblåsende Fabrikk, who designed and constructed thetransport apparatus are also gratefully acknowledged. We also gratefully acknowledge the support by the Walther Cancer Institute.

References

1 Stein, W.D. (1986) Transport and Diffusion Across Cell Membranes, Academic Press, New York.
2 Yoshikawa, K. and Matsubara, Y. (1984) J. Am. Chem. Soc. 106, 4423–4427.
3 Toshikawa, K. andd Matsubara, J. (1983) J. Am. Chem. Soc. 105, 5967–5969.
4 Yoshikawa, K., Sakabe, K., Matsubara, Y. and Oda, T. (1984) Biophys. Chem. 20, 107–109.
5 Guy, R.H. and Fleming, R. (1981) J. Colloid Interface Sci. 83, 130–137.
6 De Clercq, E. and Walker, R.T. (1986) Prog. Med. Chem. 23, 187–218.
7 De Clercq, E., Descamps, J., Verhelst, G., Walker, R.T., Jones, A.S., Torrence, P.F. and Shugar, D. (1980) J. Infect. Dis. 141, 563–574.

8 Bergstrom, D.E., Ruth, J.L., Reddy, P.A. and De Clercq, E. (1984) J. Med. Chem. 27, 279–284.
9 Ryu, E.K., Ross, R.J., Matsushita, T., MacCoss, M., Hung, C.I. and West, C.R. (1982) J. Med. Chem. 25, 1322–1329.
10 Matsushita, T., Ryu, E.K., Hong, C.I. and MacCoss, M. (1981) Cancer Res. 41, 2707–2713.
11 Rosowsky, A., Kim, S.-H., Ross, J. and Wick, M.M. (1982) J. Med. Chem. 25, 171–178.
12 Chawla, R.R., Freed, J.J., Kappler, F. and Hampton, A. (1986) J. Med. Chem. 29, 797–802.
13 Kanzawa, F., Matsushima, Y., Ishihara, J.-I., Hoshi, A., Ohba, T. and Watanabe, K. (1986) Pharamcobio-Dyn. 9, 688–693.
14 Schwendener, R.A., Supersaxo, A., Rubas, W., Weder, H.g., Hartmann, H.R., Schott, H., Ziegler, A. and Hengartner, H. (1985) Biochem. Biophys. Res. Commun. 126, 660–666.
15 Cohen, S.S. and Plunkett, W. (1975) Ann. NY Acad. Sci. 255, 269–286.
16 Plunkett, W., Lapi, L., Ortiz, P.J and Cohen, S.S. (1974) Proc. Natl. Acad. Sci. USA 71, 73–77.
17 Plunkett, W. and Cohen, S.S. (1975) Cancer Res. 35, 1547–1554.
18 Gokel, G.W. and Garcia, B.J. (1977) Tetrahedron Lett. 317–321.
19 Bergstrom, D.E. and Ogawa, M.K. (1978) J. Am. Chem. Soc. 100, 8106–8112.
20 Duval, J. and Ebel, J.P. (1964) Bull. Soc. Chim. Biol. 46, 1059–1071.
21 Moffatt, J.G. and Khorana, H.G. (1961) J. Am. Chem. Soc. 83, 649–658.
22 Sussman, J.L., Seeman, N.C., Kim, S.-H. and Berman, H.M. (1972) J. Mol. Biol. 66, 403–421.
23 Cussler, E.L. (1984) Diffusion: Mass Transfer in Fluid Systems, Cambridge University Press, Cambridge.
24 Prigonine, I. and Stengers, I. (1984) Order Out of Nature: Man's New Dialogue with Nature, Bantam Books, New York.
25 Toko, K., Nakasha, N., Iiyama, S., Yamafuji, K. and Kunitake, T. (1986) Chem. Lett. 1375–1378.
26 Dupeyrat, M. and Nakache, E. (1978) Bioelectrochem. Bioenerg. 5, 134–141.
27 Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W. (1977) Biochim. Biophys. Acta 470, 185–201.
28 Broom, A.D., Schweizer, M.P. and Ts'O, P.O.P. (1967) J. Am. Chem. Soc. 89, 3612–3622.
29 Parks, D. (1974) Adv. Drug. Res. 8, 11–81.
30 Donath, E., Herrmann, A., Coakley, W.T., Growth, T., Egger, M. and Taegr, M. (1987) Biochem. Pharma. 36, 481–487.
31 Tverdislov, V.A., El-Karadaghi, S., Bucher, D.J., Zakomirdin, J.A. and Kharitonenkov, I.G. (1984) Biochim. Biophys. Acta 776, 276–280.
32 Israelachvili, J.N. and McGuiggan, P.M. (1988) Science 241, 795–800.
33 Pedley, T.J. (1983) Quart. Rev. Biophys. 16, 115.